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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/12, C07K 13/00

A2

(11) International Publication Number: WO 93/16180

(43) International Publication Date: 19 August 1993 (19.08.93)

(21) International Application Number: PCT/EP93/00382

(22) International Filing Date: 17 February 1993 (17.02.93)

(30) Priority data:
RM92A000100 17 February 1992 (17.02.92) IT

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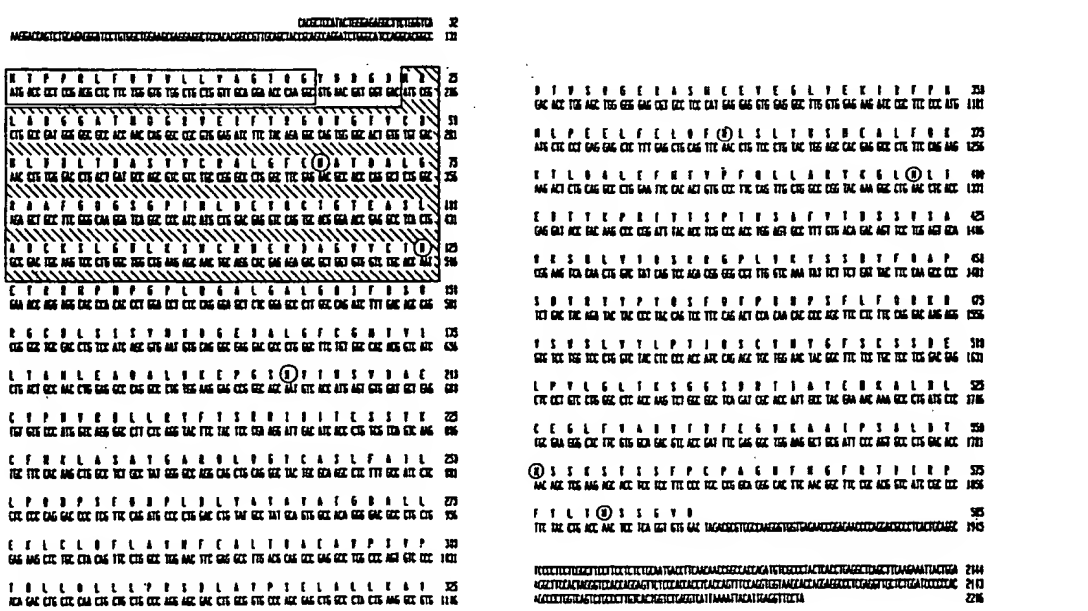
(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: SEQUENCES FOR A 90K TUMOR-ASSOCIATED ANTIGEN, IMMUNOREGULIN-95 (IR-95)



(57) Abstract

This invention provides substantially purified tumor-associated 90K antigen, or fragment(s) thereof, especially from: the culture fluid of the human breast cancer cell line, CG-5; the serum of a breast cancer patient; or the ascitic fluid from an ovarian cancer patient. The native antigen, which has a molecular weight of about 95,000 daltons, is present as a high molecular weight complex. The purification and characterization of the antigen is provided as well as uses thereof. The nucleotide sequences which encode the 90K antigen, or fragment(s) thereof, vehicles containing the genetic sequence, hosts transformed therewith, and production of the antigen, or fragments thereof, by the transformed host are also provided.

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SEQUENCES FOR A 90K TUMOR-ASSOCIATED ANTIGEN, IMMUNOREGULIN-95 (IR-95)

Background of the Invention

Field of the Invention

The invention, in the field of molecular and cellular biology, relates to the purification and characterization of the 90K tumor-associated antigen (IR-95), to genetic sequences which encode the 90K antigen, to the cloning and expression of this antigen, to its production and to uses thereof.

Background Information

Antigens shed or secreted by tumor cells have been reported in the serum of patients with different forms of cancer. Immunoassays of some of these molecules show that they have potential use as diagnostic/prognostic indicators and for therapeutic surveillance. Some of the recognized antigens include: CA125 for ovarian cancer (Bast *et al.*, *N. Engl. J. Med. 309*:883-887 (1983)); MOV2 for ovarian cancer (Miotti *et al.*, *Cancer Res. 45*:826-832 (1985)); CA15-3 for breast cancer (Hilkens *et al.*, *Cancer Res. 46*:2582-2587 (1986)); CA19-9 for gastrointestinal cancer (Koprowski *et al.*, *Science 212*:53-55 (1981)); carcinoembryonic antigen (CEA) for gastrointestinal cancer (Golp *et al.*, *JAMA 234*:1331-1334 (1968)); and CA50 for gastrointestinal cancer (Holmgren *et al.*, *Br. Med. J. 288*:1479-1482 (1984)). However, none of these tumor antigen serodetection assays have been sensitive enough to permit the early detection of occult cancer, or the reoccurrence or metastases thereof.

While these antigens are mostly expressed on the surface of tumor cells, some are secreted into the circulation of patients. This last category of antigens may prove useful for the serodetection, prognosis and assessment of tumor load and cancer development.

Monoclonal antibodies (MAbs) which detect tumor-associated antigens have been reported. For example, MAbs against circulating breast cancer-associated antigens have been obtained. One such MAb, SP-2, identified a cytoplasmic antigen, termed the 90K antigen (a.k.a. ImmunoRegulin-95 or IR-95), which is expressed in more than 80% of breast cancers (Iacobelli *et al.*, *Cancer Res. 46:*3005-3010 (1986)).

Approximately 50% of the patients with breast cancer, 40% of the patients with gastrointestinal malignancies, and 30% of the patients with gynecological malignancies had elevated serum levels of the 90K antigen (Iacobelli *et al.*, *Breast Cancer Res. & Treat. 11*:19-30 (1988)). More importantly, the assay of the present invention has demonstrated that the percentage of patients showing elevated serum levels is greater for individuals with metastatic disease and that the 90K serum changes correlated with cancer progression (Iacobelli *et al.*, *Breast Cancer Res. & Treat. 11*:19-30 (1988); Scambia *et al.*, *Anticancer Res. 8:*761-764 (1988); Benedetti-Panici *et al.*, *Gynecol. Oncol. 35*:286-289 (1989)). Since the 90K antigen is distinct from other circulating antigens such as CA 15-3, CEA, and CA 125 (Iacobelli *et al.*, *Breast Cancer Res. & Treat. 11*:19-30 (1988); Benedetti-Panici *et al.*, *Gynecol. Oncol. 35*:286-289 (1989)), it may represent an additional useful diagnostic tool for the surveillance of breast cancer and other malignant diseases.

Homology in the region of amino acids 35-80 of the 90K antigen is found with the type I macrophage scavenger receptor (Kodama *et al.*, *Nature 343*:531 (1990)); sea urchin speract receptor (Dangott *et al.*, *Proc. Natl. Acad. Sci. USA 86*:2128 (1989)); and human lymphocyte glycoprotein T1/Leu-1 (Jones *et al.*, *Nature 323*:346 (1986)).

The 90K antigen is referred to in European Patent Application Number 91830153.2 filed on April 17, 1991 (Publication Number 0 453 419 A2). An antigen with the same 15 amino acid terminal sequence is referred to in PCT Application Number PCT/US85/02132 which was filed on 30 October 1985 and has International Publication Number WO 86/02735. This PCT

application claims priority to U.S. applications 667,521 and 785,177 which were filed on November 2, 1984 and October 7, 1985. However, no studies have specifically elucidated the physicochemical and immunochemical properties of this antigen. Therefore, it is important to purify and characterize the SP-2-reactive 90K antigen.

Summary of the Invention

The application is drawn to the purification and characterization of the 90K tumor-associated antigen from: the culture fluid of a human breast cancer cell line, CG-5; the serum of a breast cancer patient; and the ascitic fluid of an ovarian cancer patient. A purification procedure is provided which results in at least a 50,000 fold purification of the 90K tumor-associated antigen from the three different sources. The native antigen is a glycoprotein and has an apparent molecular weight of about 95,000 daltons and is present as a high molecular weight complex with similar electrophoretic profiles and immunoreactivity from all three sources.

The invention is further drawn to the amino acid sequence of the 90K antigen and to the genetic sequence which encodes the 90K antigen. Therapeutic and diagnostic uses of the 90K antigen are also provided.

Brief Description of the Drawings

FIGURE 1. The nucleotide and amino acid sequence of the 90K protein (SEQ ID NO:1 and SEQ ID NO:2, respectively). The signal peptide is boxed, the SRCR homology region is shaded, and potential asparagine-linked glycosylation sites are circled.

FIGURE 2. Sepharose CL-6B column chromatography of the 90K antigen which had been isolated from CG-5 tissue culture fluid (——); the serum of a breast cancer patient (.....); and the ascitic fluid of an ovarian cancer patient (--). Fractions were assayed for 90K activity by

immunoradiometric assay (IRMA). The arrow indicates the elution volume of Dextran blue 2000.

FIGURE 3. Density gradient centrifugation of the 90K antigen. Purified 90K from CG-5 culture fluid (——), the serum of a breast cancer patient (.....), the ascitic fluid from an ovarian cancer patient (--), and unfractionated serum from a breast cancer patient (–·–) were subjected to equilibrium ultracentrifugation in cesium chloride. Fractions were assayed for 90K activity by IRMA and their densities were determined by weighing a known volume of each. The arrow indicates the buoyant density of β-galactosidase.

FIGURE 4. Molecular weight determination of the 90K antigen. (Figure 4A): Immunoprecipitates of radioactive 90K antigen from human breast cancer cells. Aliquots (200,000 cpm thrichloroacetic acid precipitable) of (^{35}S)methionine-labeled culture fluid were immunoprecipitated with MAb SP-2 (lanes a-e) or MAb against alfa-fetoprotein (lane f), and were analyzed by SDS:PAGE in the presence (lanes a-c, and e) or absence (lane d) of 2-mercaptoethanol, followed by fluorography. Lane a contained CG-5 cells. Lane b contained MCF7 cells. Lane c contained T47D cells. Lane d contained T47D cells. Lane e contained tissue culture fluid from CG-5 cells after the cells had been exposed to tunicamycin but before (^{35}S)methionine labeling. (Figure 4B): SDS:PAGE analysis of 90K antigen purified from: CG-5 culture fluid (lane a, 620 units); serum from a breast cancer patient (lane b, 920 units); and ascitic fluid from an ovarian cancer patient (lane c, 700 units). The gels were silver stained. The molecular weight standards were: phosphorylase b (Mr 97,000) and BSA (Mr 66,000).

FIGURE 5. PAGE and western blot analyses of purified 90K antigen from: CG-5 culture fluid (lanes a and d); the serum of a breast cancer patient (lanes b and e); and the ascitic fluid from an ovarian cancer patient (lanes c and f). Purified 90K antigen was analyzed on the 4-20% gradient gel containing 0.1% NP-40. Lanes a-c were silver stained. Lanes d-f proteins

were electroblotted onto a nitrocellulose membrane. The molecular weight standards were: β-galactosidase (Mr 540,000) and BSA (Mr 66,000).

FIGURE 6. The effect of enzymatic digestion on the 90K antigen. (Figure 6A): Purified 90K from CG-5 culture was digested with various proteases and was analyzed on 9% SDS:PAGE followed by silver staining. (Figure 6B): The binding of (^{125}I)labeled SP-2 to digested 90K relative to untreated control is displayed. For both Figures 6A and 6B: lane a was purified 90K control; lane b was pronase-treated 90K antigen; lane c was papain-treated 90K antigen; lane d was trypsin-treated 90K antigen; and lane e was chymotrypsin-treated 90K antigen. For Figure 6B: lane f was neuraminidase-treated 90K antigen; lane g was fucosidase-treated 90K antigen; lane h was chondroitinase ABC-treated 90K antigen; lane i was α-galactosidase-treated 90K antigen; and lane l was β-galactosidase-treated 90K antigen.

FIGURE 7. Plasmid map of CMV-IR95.

FIGURE 8. Plasmid map of CMVNEO-IR95.

FIGURE 9. An autoradiogram of immunoprecipitates of the first three stable clones in human mammary carcinoma BT20 cells.

FIGURE 10. SDS-PAGE of ^{35}S-methionine labeled transiently expressed IR-95 in 293 cells transfected with plasmid pCMV-IR-95.

FIGURE 11. Percentage of cell lysis versus various IR-95 concentrations.

Detailed Description of the Invention

The present invention provides a substantially purified tumor-associated antigen which has an apparent molecular weight of approximately 95 kilodaltons (K) and is designated the 90K antigen (a.k.a. ImmunoRegulin-95 or IR-95). The concentration of this tumor-associated antigen is elevated in the serum of patients with cancer, such as breast cancer, gastrointestinal

malignancies, and gynecological malignancies, and also in patients with the human immunodeficiency virus (HIV).

The 90K antigen reacts with MAb SP-2 which was produced by immunizing mice with proteins that had been released into tissue culture fluid by human MCF-7 breast cancer cells maintained therein. The hybridoma cell line which produces MAb SP-2 was deposited according to rules 28 and 28a of the European Patent Convention on April 12, 1991 at the Institut Pasteur, Collection Nationale de Cultures de Microorganisms, 28 Rue de Docteur Roux, 75724 Paris Cedex 15, France. This deposit has been given the Accession Number I-1083. The cells were found to be viable on April 22, 1991. Utilizing MAb SP-2 to detect the antigen, it has been demonstrated that low levels of 90K are present in normal subjects, whereas antigen levels up to 100 times that of normal levels have been detected in 50% of patients with breast cancer. The 90K antigen has also been detected in the sera of patients having carcinomas of non-breast origin, including carcinomas of the ovary, endometrium, and colon.

In accordance with the invention, a 90K tumor-associated antigen or determinant can be isolated from a sample containing the antigen. Any sample that contains the antigen may be utilized as a starting material according to the methods described in the invention. The 90K tumor-associated antigen of the present invention is a glycoprotein found in the tissues and sera of patients with breast cancer and other malignant neoplasms, and with HIV infection. Therefore, it is possible to isolate the 90K protein from: the plasmas or serum of humans or other animals; naturally occurring tumor cell lines from humans or other animals which naturally produce the 90K protein; immortal cell lines from humans or other animals which do not endogenously produce the 90K protein but which have been made to do so by having been transfected with a 90K expression plasmid; and cell lines from humans or other animals which do not endogenously produce the 90K protein, and that are capable of growing in the absence of serum additives (such as U 937 cells) and which have been transfected with the 90K gene. For example, any source of the antigen is

contemplated for use in this invention including, but not limited to: the culture fluid of the human breast cancer cell line, CG-5; serum from patients with breast cancer; and ascitic fluid from patients with ovarian cancer. As used herein, the sample containing the antigen will be referred to simply as "the sample" and is intended to include any 90K antigen-containing sample.

Generally, a four-step procedure to purify the 90K antigen is utilized to practice this invention. The procedure comprises ammonium sulfate precipitation, gel filtration chromatography, ion-exchange chromatography, and adsorption to a MAb SP-2 affinity matrix. However, it is recognized that some variation in the procedure may still result in the production of highly purified 90K antigen.

The purification procedure used to isolate the 90K antigen from a sample is summarized in Table 1. After centrifugation of the sample, the protein was precipitated by adding solid ammonium sulfate and allowing the sample to stand overnight at 4°C. Protein precipitates were collected by centrifugation. At each step of purification, the total protein was determined and the antigen was quantified by IRMA. Virtually all 90K activity was recovered after ammonium sulfate precipitation, resulting in about a four-fold enrichment thereof.

The ammonium sulfate-precipitated antigen was next subjected to size exclusion chromatography. The 90K antigen was constantly found in a large peak eluting immediately behind the void volume of the column, implying that it is a high molecular weight complex. Minor reactivity peaks of lower molecular weight were also inconsistently observed which were probably due to degradation products.

The high molecular weight peak was further purified by DEAE-cellulose chromatography. The 90K antigen eluted from the column at a NaCl concentration of about 0.25M NaCl.

The final purification was accomplished by immunoaffinity adsorption on Sepharose coupled to MAb SP-2. The coupling was done by the method

of Schneider *et al.* (*J. Biol. Chem. 257:*10766-10769 (1982)). Bound 90K antigen was eluted with buffer, preferably 3M $MgCl_2$.

The purification procedure resulted in a substantially purified 90K antigen. By substantially purified is meant that the purification of the 90K antigen, as described herein, resulted in at least a 50,000-fold, and generally about 50,000- to about 80,000-fold purification of the 90K antigen.

The invention is thus drawn to substantially purified 90K antigen having an apparent molecular weight of approximately 95,000 daltons, as well as to antigenic determinant-containing fragments, and other fragments thereof. The invention is also drawn to naturally occurring fragments of the 90K antigen. The invention is further drawn to unglycosylated moieties of the 90K antigen.

As used herein, polypeptides containing immunologically cross-reactive antigenic determinants means polypeptides having a common antigenic determinant with which a given antibody will react. Such polypeptides include the glycosylated and unglycosylated moieties of the 90K antigen and fragments thereof, as well as synthetic polypeptides, or fragments thereof, and antibodies which are anti-idiotypic towards the active determinant(s) of the 90K protein. It has been demonstrated that anti-idiotypic reagents are useful as diagnostic tools for the detection of antigens carrying sites which are immunologically cross-reactive with those on antibodies (Potocnjak *et al.*, *Science 215:*1637-1639 (1982)).

Once the antigen has been purified, monoclonal and polyclonal antibodies can be generated to it using standard techniques which are well known to those of skill in the art (Klein, J., *Immunology: The Science of Cell-Noncell Discrimination*, John Wiley and Sons, New York, New York, USA (1982); Kenneth *et al.*, *Monoclonal Antibodies, Hybridoma: A New Dimension in Biological Analyses*, Plenum Press, New York, New York, USA (1980); Campbell, A., "Monoclonal Antibody Technology," In: *Laboratory Techniques in Biochemistry and Molecular Biology*, Vol. 13 (Burdon *et al.*, eds.), Elsevier, Amsterdam, The Netherlands (1984); and Eisen, H.N., In:

Microbiology, 3rd Edition (Davis *et al.*, eds.), Harper & Row, Philadelphia, PA, USA (1980)).

Of special interest to the invention are antibodies to the 90K antigen or its derivatives which are produced in humans, or are "humanized" (i.e., non-immunogenic in a human) by recombinant DNA or other technology. Humanized antibodies may be produced, for example, by replacing an immunogenic portion of an antibody with a corresponding, nonimmunogenic, portion (i.e., chimeric antibodies). See, Robinson *et al.*, International Patent Publication PCT/US86/02269; Akira *et al.*, European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison *et al.*, European Patent Application 173,494; Newberger *et al.*, PCT Application WO86/01533; Cabilly *et al.*, European Patent Application 125,023; Better *et al.*, *Science 240*:1041-1043 (1988); Liu *et al.*, *Proc. Natl. Acad. Sci. USA 84*:3439-3443 (1987); Liu *et al.*, *J. Immunology 139*:3521-3526 (1987); Sun *et al.*, *Proc. Natl. Acad. Sci. USA 84*:214-218 (1987); and Shaw *et al.*, *J. Natl. Cancer Inst. 80*:1553-1559 (1988)). General reviews of humanized chimeric antibodies are provided by Morrison, S.L., (*Science 229*:1202-1207 (1985)) and Oi *et al.*, (*BioTechniques 4*:214 (1986)).

The purified 90K protein can be sequenced using methods which are well known to those of skill in the art. Initial sequencing of the terminal amino acid sequence of the 90K protein has revealed the following amino acid sequence (SEQ ID NO:3): Val Asn Asp Gly Asp Met Arg Leu Ala Asp Gly Gly Ala Thr Asn Gln Gly Arg Val Glu Ile Phe. An analysis of the amino acid composition of the 90K antigen is found in Table 4. Further characterization of the 90K antigen is provided in Table 2 which gives the effects of chemical and physical treatments on 90K activity.

It is generally recognized that having the amino acid sequence of a protein enables one to make oligonucleotide probes which can be used to identify clones of the protein. Thus, hybridization with the appropriate nucleic acid probe will identify clones containing the nucleotide sequence coding for the 90K antigen.

As used herein, "DNA construct" means any DNA sequence which has been created synthetically or through recombinant DNA technology. "DNA constructs" include, but are not limited to, synthetic oligonucleotides, vectors and vectors containing inserts.

Particular nucleotide probes which are useful for identifying the 90K antigen genes can be constructed from knowledge of the amino acid sequence of the 90K protein. The sequence of amino acid residues and the peptide is designated herein using either the commonly employed 3-letter or single-letter designations therefor. A listing of these three- and one-letter designations may be found in textbooks such as Lehninger, A., *Biochemistry*, Worth Publishers, Inc., New York, New York, USA (1975) and subsequent volumes thereof.

The N-terminal sequence of the first twenty-two amino acids enabled the synthesis of a 66 nucleotide long oligonucleotide which was utilized as a probe to screen a cDNA library from MCF-7 cells. In this manner, the inventors have completed the molecular cloning and have determined the complete cDNA sequence of the 90K antigen.

The invention comprises the amino acid sequence of the 90K antigen, the genetic sequences coding for the antigen, vehicles containing the genetic sequence, hosts transformed therewith, 90K protein production by transformed host expression, purification of the 90K protein from a sample, and utilization of the 90K antigen.

Nucleotide and amino acid sequences for the 90K protein are shown in Figure 1 (SEQ ID NO:1 and SEQ ID NO:2, respectively). It is understood that modifications of the specified amino acid and nucleic acid sequences are encompassed by the present invention. As used herein, the term "modification" is intended to mean any substitution, addition or deletion of one or more amino acids of the polypeptide fragment or nucleotides of the nucleotide sequence. These modifications may be made by manipulating the amino acid sequence itself or by modification of the nucleic acid sequence which is then used to synthesize the peptide.

Changes in the nucleic acid sequence can be effected by mutating the DNA, usually by site-directed mutagenesis. The techniques of site-specific mutagenesis are well known to those of skill in the art, (see, for example, Adelman *et al., DNA 2*:183 (1983); Smith, M., *Ann. Rev. Genetics 19*:423 (1985)). Mutations include, for example, substitutions, additions, or deletions of nucleotide(s), provided that the final construct has the desired biologic activity. The nucleic acid changes must not place the sequence out of reading frame and preferably should not create complementary regions that could produce secondary mRNA structure (see EP Patent Application Publication No. 75,444).

Methods for the modification of amino acids as well as nucleic acids are known in the art. Amino acid sequence insertions include amino and/or carboxyl-terminal fusions from one residue to polypeptides of essentially unrestricted length, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions may range generally from about 1 to about 10 residues. More preferably they range from about 1 to about 5 residues.

The amino acid residues may be in their protected or unprotected form, using appropriate amino or carboxyl protecting groups. In addition, the synthesized peptides may be glycosolated or unglycosolated.

To express the 90K antigen, transcriptional and translational signals which are recognizable by an appropriate host are necessary. The cloned nucleic acid sequences encoding the 90K protein, preferably in double-stranded form, may be operably linked to sequences controlling transcriptional expression in an expression vector, and introduced into a host cell, either prokaryotic or eukaryotic, to produce recombinant 90K protein or variants thereof. Depending upon which strand of the 90K protein encoding sequence is operably linked to the sequence(s) controlling transcriptional expression, it is also possible to express 90K protein antisense RNA or variants thereof.

As used herein, "expression vehicle" means a DNA construct which is capable of directing the expression of an operably linked DNA sequence.

Expression vehicles include, but are not limited to, phage and plasmid vehicles. "Expression vehicles" typically contain one or more elements selected from the group consisting of, but not limited to, an operator, a promoter, a ribosome binding site, a translation-initiation signal and a translation terminator.

As used herein, "host cell" means any cell capable of being transformed or transfected with a DNA construct or an expression vehicle.

Expression of the 90K protein in different hosts may result in varying post-translational modifications which may alter the properties of the protein.

A nucleic acid molecule, such as DNA, is said to be "capable of expressing" a polypeptide if it contains expression control sequences which contain transcriptional regulatory information. For expression of a polypeptide, control sequences must be "operably linked" to the nucleotide sequence which encodes the polypeptide.

An operable linkage is a linkage in which a nucleotide sequence encoding a polypeptide is connected to a regulatory sequence (or sequences) in such a way as to place expression of the polypeptide encoding sequence under the influence or control of the regulatory sequence. Two DNA sequences (such as a 90K protein encoding sequence and a promotor region sequence linked to the 5′ end of the encoding sequence) are said to be operably linked if the induction of promoter function results in the transcription of the protein encoding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the expression regulatory sequences to direct the expression of the 90K mRNA, antisense RNA, or protein, or (3) interfere with the ability of the 90K template to be transcribed by the promoter region sequence. Thus, a promoter region would be operably linked to a DNA sequence if the promoter were capable of effecting transcription of that DNA sequence.

The precise nature of the regulatory regions needed for gene expression may vary between species or cell types, but generally includes 5′ non-coding

sequences involved with the initiation of transcription and translation, such as the TATA box, capping sequence, CAAT sequence, and the like. Such 5' non-coding control sequences will especially include a region which contains a promoter for the transcriptional control of an operably linked gene.

Expression of the 90K protein in eukaryotic hosts requires the use of regulatory regions, preferably eukaryotic, which are functional in such hosts. A wide variety of transcriptional and translational regulatory sequences can be employed, depending upon the nature of the eukaryotic host. The transcriptional and translational regulatory signals can also be derived from the genomic sequences of viruses which infect eukaryotic cells, such as adenovirus, bovine papilloma virus, Simian virus, herpes virus, or the like. Preferably these control signals are associated with a particular gene which is capable of a high level of expression in the host cell.

Promoters from mammalian genes which encode mRNA products capable of being translated are preferred, and especially, strong promoters such as the promoter for actin, collagen, myosin, etc., can be employed, provided they also function as promoters in the host cell. For eukaryotic promoters see generally, Hamer *et al., J. Mol. Appl. Gen. 1*:273-288 (1982); McKnight, S., *Cell 31*:355-365 (1982); Benoist *et al., Nature (London) 290*:304-310 (1981); Johnston *et al., Proc. Natl. Acad. Sci. USA 79*:6971-6975 (1982); and Silver *et al., Proc. Natl. Acad. Sci. USA 81*:5951-5955 (1984).

General methods for molecular cloning and expression can be found in Sambrook *et al., Molecular Cloning: A Laboratory Manual, 2d. ed., Vols. 1-3*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA (1989).

Transcriptional initiation regulatory signals can be selected which allow for the repression or activation of gene expression, so that expression of the operably linked genes can be modulated. The vectors of the invention may further comprise other operably linked regulatory elements, such as enhancer

sequences or DNA elements, which confer tissue or cell-type specific expression on an operably linked gene.

The purified protein and antibodies thereto as well as its genetic sequences are useful in diagnostic and therapeutic methods.

In particular, the level of the 90K antigen is useful as a diagnostic indicator for cancer, including breast, ovarian and other malignancies, viral infection, including HIV, inflammation, autoimmune disease, aging, and the like.

The 90K antigen can be assayed by a variety of methods. In serum, the 90K antigen can be assayed utilizing an enzyme-linked immunosorbent assay (ELISA) sandwich procedure. In this manner, MAb SP-2 can be utilized both as an immunoabsorbent and as an enzyme-labeled probe to detect and quantify the 90K antigen by a sandwich-type ELISA. The amount of 90K present in the sample can be calculated by reference to the amount present in a standard preparation of CG-5 cell lysate using a linear regression computer program. The assay has been previously described by Iacobelli *et al. (Breast Cancer Res. and Treatment 11*:19-30 (1988)), which reference is herein incorporated in its entirety. Overexpression of the 90K antigen would be an indicator of a disorder.

Expression levels of the 90K antigen can also be determined by measuring the levels of RNA. In this method, a nucleic acid probe can be utilized to hybridize to the RNA in the sample. Methods for hybridization are generally known to those of skill in the art (see, for example, *Nucleic Acid Hybridization, A Practical Approach*, IRL Press, Washington, D.C., USA (1985) and the references cited therein).

The 90K antigen or its genetic sequences may also be useful in therapy. Serum IR-95 levels are elevated not only in patients with cancer, but also in those affected by different physiopathological conditions (see Table 5), such as infection by HIV or other viruses, autoimmune disease, etc., all of which are characterized by a variable degree of immune deficit associated with immune activation.

In vitro experiments have also shown that the 90K antigen is able to enhance natural killer (NK) and lymphokine activated killer (LAK) cell activity of peripheral blood mononuclear cells (Figure 11).

Given the above findings, the 90K antigen or its genetic sequences may also be useful in therapy as an immunoregulatory agent. For example, patients who suffer from a particular cancer which does not induce overexpression of the 90K antigen may be treated by infusion with the 90K antigen. Furthermore, those patients with cancers that generate elevated levels of the 90K protein in their serum, may be supplied additional 90K antigen by infusion.

The 90K antigen or its genetic sequences may also be useful in gene therapy (reviewed in Miller, *Nature* 357:455-460 (June 1992). In one preferred embodiment, an expression vector containing the IR-95 coding sequence is inserted into cells, the cells are grown *in vitro* and then infused in large numbers into patients. In another preferred embodiment, a DNA segment containing a promoter of choice (for example a strong promoter) is transferred into cells containing an endogenous IR-95 in such a manner that the promoter segment enhances expression of the endogenous IR-95 gene (for example, the promoter segment is transferred to the cell such that it becomes directly linked to the endogenous IR-95 gene).

The 90K antigen or antagonists thereof can routinely be prepared as therapeutic agent(s) by one of skill in the art using standard techniques and references which are well known in the art (see, for example, *Remington's Pharmaceutical Sciences*, 18th ed., (A.R. Gennaro, Ed.), Mack Publishing Comp., Easton, PA, USA 18042 (1990), especially chapters 8 (Pharmaceutical Preparations and Their Manufacture) and 4 (Testing and Analysis), thereof).

As used herein, by "antagonist" is meant any compound that decreases the effect of the 90K antigen *in vivo* or *in vitro*.

Appropriate and optimum routes of administration can also be routinely determined by one of skill in the art. The former include the oral,

intravenous, intramuscular, subcutaneous, transdermal, *in situ* and bucal routes of administration among others.

The doses of the 90K antigen and antagonist(s) thereof which is useful as a treatment are "therapeutically effective" amounts. As used herein, a "therapeutically effective amount" means an amount of the antigen, fragment or antagonist thereof, which produces the desired therapeutic effect. This amount can be routinely determined by one of skill in the art and will vary depending upon several factors such as the particular illness from which the patient suffers and the severity thereof, as well as the patient's height, weight, sex, age, and medical history. Generally, the 90K antigen of the present invention is preferably provided at a dose of between about 5 to about 5000 mg/dose/week/patient. More specifically, one preferable dose range is from 50 to 500 mg/dose/week/patient.

For the treatment of autoimmune disease, rheumatoid arthritis, allergy, rejection of organ transplants, and other pathological situations where the immune system is activated and needs to be suppressed, a 90K antigen antagonist can be administered. The appropriate doses of the antagonist can be routinely determined by one of skill in the art as described above. Generally the antagonist(s) of the 90K antigen is preferably provided at a dose of between about 5 to about 5000 mg/dose/week/patient. More specifically, one preferable dose range is from 50 to 500 mg/dose/week/patient.

Any terms which are used herein and are not specifically defined herein are used as they would be by one of ordinary skill in the art(s) to which the invention pertains.

The Examples which follow are for illustrative purposes only and are not intended to limit the scope of the invention.

Example 1
Characterization of the 90K Antigen

Materials and Methods

Cell Lines and Reagents. CG-5, an estrogen-supersensitive variant of the MCF-7 human breast cancer cell line (Natoli *et al.*, *Breast Cancer Res. Treat.* *3*:23-32 (1983)) and other human breast cancer cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. The murine MAb SP-2 produced by hybridomas grown in pristane-primed Balb/c mice (Iacobelli *et al.*, *Cancer Res.* *46:*3005-3010 (1986)) was purified from ascitic fluid by ammonium sulfate precipitation followed by ion-exchange chromatography (Iacobelli *et al.*, *Breast Cancer Res. & Treat.* *11*:19-30 (1988)). Hybridoma cells which produce MAb SP-2 were deposited under the provisions of the European Patent Convention at the Pasteur Institute as previously described. This cell line was given the deposit number I-1083.

Purified MAb SP-2 was labeled with $Na^{125}I$ using lactoperoxidase (Thorell *et al.*, *Biochem. Biophys. Acta 251:*363 (1971)). The proteases and other enzymes were purchased from Sigma Chemical Corp., St. Louis, MO, U.S.A. Electrophoresis reagents were purchased from Bio-Rad Laboratories, Segrate, Italy. Sepharose CL-6B was purchased from Pharmacia, Uppsala, Sweden. All other reagents were of the highest purity commercially available.

Solid-Phase Radioimmunoassay. A "two-step" sandwich IRMA was developed to measure 90K activity. Polystyrene beads (6.5 mm, Precision Plastic Balls, Chicago, Illinois, USA) were coated with biotinylated MAb SP-2 by the protein-avidin-biotin-capture system (Suter *et al.*, *Mol. Immunol.* *26:*221-230 (1989)). Biotinylation of SP-2 was carried out according to the method of Guesdon *et al.* *(J. Histochem. Cytochem.* *27:*113-118 (1979)). After coating, the beads were washed extensively with 0.9% NaCl solution and were incubated with biotinylated MAb SP-2 (5 μg/ml) at room temperature for 18

hours. Coated beads were treated with a blocking solution of BSA (2 mg/ml) for 1 hour at room temperature, were washed with distilled water and were stored at room temperature until used. Beads treated in this fashion were stable for at least six months.

With each assay, 200 μl of appropriately diluted samples or standards were incubated with MAb SP-2-coated beads for 1 hour at 37°C. The beads were washed with distilled water followed by the addition of 100 μl of (^{125}I)-labeled MAb SP-2 (approximately 50,000 cpm; specific activity, 10 μCi/μg) in PBS, pH 7.4, containing 5% BSA, 0.1 mg/ml normal mouse IgG and 0.1% NaN_3 for an additional hour at 37°C. The beads were washed with distilled water and were counted in a gamma-counter. The amount of 90K was calculated by reference to the amount present in standard preparations made from a pool of sera from breast cancer patients and titered to contain 40, 20, 10, and 5 arbitrary units/ml. The simultaneous assay of 120 sera from breast cancer patients using IRMA and ELISA (Iacobelli *et al.*, *Breast Cancer Res. & Treat. 11*:19-30 (1988)) gave a correlation coefficient of 0.91 (Kendall Q test). Compared to ELISA, IRMA is approximately three times more sensitive, faster to perform, requiring less than 3 hours, and highly reproducible with an inter- and intra-assay coefficient of variation of 4%.

PAGE and Western Blotting. SDS-PAGE was performed essentially according to the method of Laemmli (*Nature 227:*680-685 (1970)) on a vertical slab gel apparatus. Samples were treated with "sample buffer" consisting of 63 mM Tris-HCl containing 1.25% SDS and 5% 2-mercaptoethanol, or 63 mM Tris HCl plus 0.25% NP-40 (Nonidet-P40, Sigma Chem. Corp., St. Louis, MO, USA). In the present study, 9% SDS-gels and 4-20% gradient gels with NP-40 were used. Gels were run at constant voltage in Tris-glycine buffer (pH 8.3) containing either 0.04% SDS or 0.1% NP-40. Protein bands were visualized with Coomassie blue R 250 or a silver stain kit (Bio-Rad Laboratories, Segrate, Italy). For immunological analysis, the gels were electroblotted onto nitrocellulose membranes at 50 V for 2 hours as described by Towbin *et al.* (*Proc. Natl. Acad. Sci. USA 76:*4350-4354 (1979)) except

that the transfer buffer did not contain methanol. The membranes were blocked with bovine skim milk, followed by incubation with MAb SP-2 (10 μg/ml) for 2 hours at room temperature. The membranes were washed thoroughly with PBS and were stained with an Extravidin-biotin Staining Kit (Sigma Chemical Corp., St. Louis, MO, U.S.A.) according to the manufacturer's instructions.

Radiolabeling of Cells and Immunoprecipitation. For metabolic labeling, 2 x 10^6 cells were incubated at 37°C for 6 hours in DMEM containing 250 μCi/ml (^{35}S)methionine (specific activity: 1500 Ci/mmole; The Radiochemical Centre, Amersham, U.K.). Culture fluids containing the radioactive proteins were pre-clarified as described by Iacobelli *et al.* *(Cancer Res. 46*:3005-3010 (1986)), and were incubated with MAb SP-2 coated polystyrene beads at 4°C for 16 hours. The beads were washed with distilled water and were extracted with 100 μl of SDS-sample buffer for 30 min at 50°C. The extracts were run on SDS:PAGE. As controls, aliquots of culture fluid were incubated with polystyrene beads that had been coated with a MAb against alpha-fetoprotein (Sorin Biomedica, Saluggia, Italy). (^{35}S)methionine-labeled protein bands were visualized by fluorography. In some experiments cells were labeled in the presence of 5 μg/ml of tunicamycin (Sigma Chemical Corp., St. Louis, MO, U.S.A.). Tunicamycin was added to the cells 2 hours before the addition of (^{35}S)methionine.

90K Purification. (a) CG-5 Tissue Culture Fluid. CG-5 cells (Natoli *et al., Breast Cancer Res. Treat.* 3:23-32 (1983)) were grown in DMEM supplemented with 3% FCS using Cell Factory plastic chambers (Nunc, Roskilde, Denmark). When the cells became confluent (5 to 7 days), the culture fluid was collected. Then fresh medium was added and collected at 24 hour intervals for an additional 3 to 4 days. The concentration of 90K antigen produced under these conditions ranged from 100 to 400 units/ml. Pooled culture supernatants (10 to 20 liters) were centrifuged at 4000 x g (10 min at 4°C) followed by a 10-fold concentration using a Minitan apparatus (Millipore Corp., Bedford, MA, USA). Solid ammonium sulfate was slowly added to

reach 43% saturation and, after standing overnight at 4°C, protein precipitates were collected by centrifugation at 10,000 x g (15 min at 4°C). The precipitates were stored frozen at -20°C under which conditions the 90K activity was stable for at least 2 months. (b) Human serum. Whole serum from a patient with advanced breast cancer which had been titered to contain high concentrations of 90K by IRMA, was clarified by centrifugation at 10,000 x g for 20 min, then was diluted 1:1 with PBS and was fractionally precipitated with ammonium sulfate as described above for tissue culture fluid. (c) Ascitic fluid. This was obtained by paracentesis from a patient with advanced ovarian carcinoma. The fluid was clarified by centrifugation at 10,000 x g for 20 min and was precipitated with ammonium sulfate as above.

The ammonium sulfate precipitates were dialyzed extensively against PBS and were applied to a Sepharose CL-6B column (4.2 x 85 cm). They were equilibrated and eluted with PBS-0.5 M NaCl, pH 8.1, at a flow rate of 18 ml/hour. Five ml fractions were collected and were assayed for 90K by IRMA. The protein was quantified by the method of Bradford (*Anal. Biochem.* *72:*248-254 (1976)). Fractions containing 90K activity were pooled, dialyzed against 0.005 M Na-phosphate buffer, pH 7.4, and were applied to a DEAE-cellulose column (2 x 8 cm) equilibrated in the same buffer. The column was washed extensively with buffer and the adsorbed proteins were eluted using a stepwise sodium chloride gradient (0.062 to 1.0 M). Fractions containing 90K activity were pooled and mixed with MAb SP-2-conjugated Sepharose CL-4B (4 mg antibody/ml resin) at a volume ratio of 8:1 (sample:resin). MAb SP-2 was coupled to Sepharose by the method of Schneider *et al.* (*J. Biol. Chem.* *257:*10766-10769 (1982)). The mixture was rotated overnight at 4°C. The 90K antigen was eluted with 3 M $MgCl_2$.

Density Gradient Centrifugation. Centrifugation of the 90K antigen isolated from CG-5 tissue culture fluid, the serum of a patient with breast cancer, or ascitic fluid from a patient with ovarian cancer, after desorption from the affinity matrix, was performed in 5 ml of a CsCl isopicnic density gradient. The antigen was dissolved in a CsCl solution in PBS with a starting

density of 1.4 g/ml, and the gradients were formed by centrifugation in a Beckman SW 50.1 rotor at 145,000 x g for 72 h at 4°C. Fractions (0.25 ml) were collected, diluted 1:10 with PBS and were assayed for antigenic activity using 90K IRMA. The density of each fraction was determined by weighing a known volume thereof.

Biochemical Characterization of the Antigen. This was performed directly on antigen seeded on microtiter plates. Microplates (Dynatecs) were coated with 50 μl of purified 90K (100 ng/ml of 0.05 M carbonate buffer, pH 9.6) and were incubated overnight.

(a) Chemical Treatment. Methanol treatment was carried out at 4°C for 30 min. Denaturation was performed with either urea 6 M and guanidine-HCl 6 M or 1% SDS at 45°C for 1 hour. Periodate oxidation was performed for 1 hour at room temperature with 10, 20, 30, 40, 50 mM $NaI0_4$ in acetate buffer (50 mM, pH 4.5) in the dark according to Stahl *et al.* (*Proc. Natl. Acad. Sci. USA 73:*4045-4049 (1976)). Reduction was performed with dithiothreitol (10 mM in 50 mM Tris, pH 8.1) or 5% 2-mercaptoethanol at 37°C for 1 hour. Alkylation was performed with 20 mM iodacetic acid at 30°C for 30 min.

(b) Proteolytic Enzymes. Antigen-coated microplates were exposed for 90 min at 37°C to trypsin (2 mg/ml), chymotrypsin (2 mg/ml), or pronase (19 mg/ml) in 50 mM Tris-2mM $CaCl_2$, pH 8.1, or to papain (0.2 mg/ml) in 50 mM cysteine-HCl, pH 6.0. In parallel experiments, aliquots of purified 90K were digested with the same proteases, were mixed with an equal volume of SDS sample buffer, and were separated by SDS:PAGE followed by silver staining.

(c) Exoglycosidases. Microplates were exposed to either neuraminidase, fucosidase, α-glucosidase and β-glucosidase in 50 mM acetate buffer, pH 5.0, or to chondroitinase ABC in 250 mM Tris, 176 mM CH_3COONa, 250 mM NaCl, pH 8.0. Incubations were carried out at 37°C for 90 min. The concentrations of exoglycosidases were chosen to ensure complete digestion of the oligosaccharide residues. This was verified in

separate experiments in which the appropriate substrates were shown to be completely hydrolyzed as detected by thin-layer chromatography.

After treatment, microplates were washed and blocked with 1% gelatin in PBS. Fifty μl of (^{125}I)labeled MAb SP-2 (approximately 50,000 cpm) were added to each well and were incubated at 37°C for 1 hour. After 3 washes with PBS, the bound radioactivity was counted in a gamma-counter. Control wells were incubated with dilution buffers under the same conditions.

Amino Acid Analysis. Purified 90K was electrophoresed through a 9% SDS polyacrylamide gel under reducing conditions using a Minigel apparatus. Proteins were electroblotted to polyvinylidene difluoride membrane (Immobilon; Millipore Corp., Bedford, MA, USA), were stained with Amido Black 10B (Sigma Chem. Co., St. Louis, MO), and the bands were excised. For amino acid analysis, 3-4 bands, for a total of approximately 50 μg of 90K (as judged by staining intensity), were hydrolyzed under vacuum in 6N HCl at 110°C for 22 hours. After hydrolysis, the amino acids were analyzed on a Beckman analyzer using a pH gradient system (Hirs, C.H.W., In: *Methods of Enzymol. 91*:3-8, Academic Press, New York, New York, USA (1983)).

Results

Purification of the 90K Antigen. The purification procedure used to isolate the 90K antigen from CG-5 tissue culture fluid, serum from a breast cancer patient, and ascitic fluid from an ovarian cancer patient is summarized in Table 1. At each step of purification, the total protein was determined, and the antigen was quantified by IRMA. Virtually all 90K activity was recovered in the 43% ammonium sulfate precipitate, resulting in about 4-fold enrichment. This step removed the large majority of albumin present in the initial preparation. Ammonium sulfate precipitated-antigen was next subjected to size exclusion chromatography using a Sepharose CL-6B column (Figure 2). The 90K from all three sources was constantly found in a large peak eluting immediately behind the void volume of the column, implying that it is a high

molecular weight complex. Minor reactivity peaks of lower molecular weight were inconsistently observed which could have been due to degradation products. Low molecular weight proteins found at the end of elution were unreactive.

Treatment of the samples with either 6 M urea or 6 M guanidine-HCl before chromatography gave identical elution profiles (not shown). The high molecular weight peak (corresponding to fractions 21 to 28 of Figure 2) was further purified by DEAE-cellulose chromatography. The 90K antigen obtained from each of the three different sources eluted from the column at a NaCl concentration of 0.25 M (data not shown).

The final purification was accomplished by immunoaffinity on Sepharose CL-4B coupled to MAb SP-2. Bound activity was eluted with 3M $MgCl_2$. Other eluting buffers which were used, such as glycine (pH 2.4), 1 M NaOH (pH 11.2), and 3M KSCN were less effective in antigen elution. Based on specific activity (units/μg protein), the purification of the 90K antigen from CG-5 tissue culture fluid, serum from a breast cancer patient, and ascitic fluid from an ovarian cancer patient were 84,300, 52,277 and 83,380-fold, respectively. These specific activities were calculated by measuring the 90K immunoreactivity in the 3 M $MgCl_2$ eluate from the affinity matrix with IRMA and determining the amount of protein by comparing the silver staining intensity of the 90K band on SDS:PAGE gels with BSA standards of known concentrations.

Analysis of Purified 90K by Density Gradient Centrifugation. Samples of antigen which had been desorbed from the MAb SP-2 affinity matrix were subjected to density gradient centrifugation. This procedure did not reveal a different average buoyant density for the antigen obtained from the three different sources. The buoyant density ranged from between 1.28 g/ml to 1.31 g/ml (Figure 3). Moreover, the 90K antigen in unfractionated serum from a patient with breast cancer produced essentially an identical density profile, indicating that the 90K antigen isolated by our purification procedure did not represent a subset of the original antigen.

PAGE and Immunoblotting Analyses of the 90K Antigen Isolated from Different Sources. In agreement with previous data (Iacobelli *et al.*, *Cancer Res.* *46*:3005-3010 (1986)), the 90K antigen released into the tissue culture fluid of (^{35}S)methionine-labeled CG-5 cells and other breast cancer cell lines migrated as a single band with an apparent molecular weight of approximately 95,000 daltons as revealed by SDS:PAGE (Figure 4A). The mobility of (^{35}S)methionine-labeled antigen was identical under reducing or nonreducing conditions (with or without 2-mercaptoethanol) (Figure 4A, lane a vs. lane d) suggesting that the protein does not contain interchain disulfide bonds. Moreover, tunicamycin treatment of CG-5 cells before labelling with (^{35}S)methionine did not alter the electrophoretic mobility of the 90K antigen in the cell culture fluid (Figure 4A, lane c).

Figure 4B compares the electrophoretic mobility on SDS:PAGE of 90K purified from CG-5 tissue culture fluid, the serum of a breast cancer patient, and ascitic fluid from an ovarian cancer patient. Silver staining for protein clearly showed a major band with an apparent molecular weight of approximately 95,000 daltons. The 95K band also stained with Coomassie blue but not with periodic acid-Schiff carbohydrate staining (data not shown). Co-electrophoresis of the purified 95K antigen from the serum of a breast cancer patient detected by silver staining and of (^{35}S)methionine-labeled immunoprecipitates from CG-5 culture fluid detected by fluorography, gave superimposable 95K bands (data not shown).

Western blot analysis of the purified 90K antigen transferred from 4-20% polyacrylamide gel containing 0.25% NP-40 but not SDS, demonstrated the presence of similar immunoreactive diffuse bands with similar mobility from all three sources (Figure 5). By contrast, immunoblotting of the 90K antigen transferred from SDS-polyacrylamide gels revealed very low MAb SP-2 immunoreactivity (data not shown). These data correlate with the Sepharose CL-6B elution profiles (Figure 2) and indicate that native 90K antigen isolated from different sources exists as a high

molecular weight complex which is likely to be composed of Mr 95,000 subunits.

Amino Acid Analysis of 90K. Table 4 shows that the 90K antigen purified from CG-5 tissue culture fluid, the serum of a breast cancer patient, and the ascitic fluid from an ovarian cancer patient have similar amino acid compositions. The antigen was relatively rich in glutamic acid/glutamine, serine, and leucine. Moreover, the NH_2-terminal sequence of the first 20 amino acids revealed a strong similarity among the antigens obtained from the three different sources. This sequence was not found in several protein data-bases such as Genebank and EMBL.

Nature of the 90K Determinant. The biochemical nature of the determinant carried on the 90K antigen was investigated using several chemical and enzymatic treatments. As Table 2 shows, exposure to methanol strongly reduced the immunoreactivity of the 90K determinant as did exposure to 6 M guanidine-HCl, 6 M urea, 1% SDS, lyophilization and heat. Neither reduction with dithiothreitol and 2-mercapoethanol, nor alkylation with iodoacetamide or treatment with the nonionic detergents NP-40, Tween 20, and Triton X-100 (Sigma Chem. Co., St. Louis, MO) significantly affected 90K immunoreactivity. Exposure to sodium-m-periodate had only marginal effect at high concentrations (50 mM).

To investigate the sensitivity of the 90K antigen to proteases, purified 90K was incubated with trypsin, chymotrypsin, pronase, or papain, and then was analyzed by SDS:PAGE followed by silver staining. As shown in Figure 6A, all the tested proteases appeared to completely digest 90K. Analysis of residual SP-2 antibody binding confirmed that more than 80% of the initial 90K activity was lost after pronase or papain exposure whereas digestion with trypsin or chymotrypsin appeared to be less effective (Figure 6B).

Treatment with exoglycosidases did not affect 90K immunoreactivity (Figure 6B). In fact, there was an increase in the ability of the immobilized antigen to bind (^{125}I)labeled MAb SP-2 following treatment with

neuraminidase and β-galactosidase. This suggests that removal of terminal carbohydrate moieties may increase access of MAb SP-2 to the 90K determinant.

Discussion

MAb SP-2 reacts with an antigenic determinant which has been termed the 90K antigen on the basis of its apparent molecular weight of 95,000 daltons (Iacobelli *et al.*, *Cancer Res.* *46*:3005-3010 (1986)). Here, we have described the purification of the 90K antigen from CG-5 culture fluid, the serum from a human breast cancer patient, and ascitic fluid from an ovarian cancer patient. We have found that the native 90K from each of these sources exists as a high molecular weight complex that was readily dissociated into a single 90,000 daltons species upon SDS:PAGE analysis. This suggests that the native protein represents an oligomer of several minimal subunits of 90,000 daltons. Interestingly, 90K antigen derived from each of the three sources exhibits similar behavior on size exclusion and ion-exchange chromatography, PAGE and Western blotting analyses, as well as buoyant density ultracentrifugation. Moreover, the antigen isolated from each of the three sources has similar amino acid composition and NH_2-terminal amino acid sequence. This indicates that the 90K antigen obtained from established long-term cancer cell lines and directly from cancer patient's serum or ascitic fluid have very similar physicochemical and immunochemical properties.

Chemical and physical treatments of the 90K antigen were undertaken to better understand the nature of the determinant recognized by MAb SP-2. Protease digestion of the 90K antigen markedly reduced the antibody binding, providing evidence that the peptide portion of the antigen is involved in the determinant. Moreover, treatments known to denaturate most proteins also greatly reduced antibody binding, thus providing further evidence that MAb SP-2 binds to a conformational peptide determinant. Furthermore, dissociation of the oligomeric structure of the antigen into subunits upon SDS:PAGE

resulted in the nearly complete loss of SP-2 binding activity. These results strongly indicate that the MAb SP-2 defined determinant is proteinaceous in nature and that antibody binding is dependent upon the conformational integrity of the whole antigen molecule. However, this is not a unique characteristic of the 90K antigen as other tumor-associated antigenic determinants, such as those recognized by MAb OC 125 (Davis *et al.*, *Cancer Res. 46*:6143-6148 (1986)), B72.3 (Johnson *et al.*, *Cancer Res. 46:*850-857 (1986)), and C 3 (Zhang *et al.*, *Cancer Res. 49:*6621-6628 (1989)), seem to be composed of, at least in part, conformationally dependent peptide.

Previously, a number of tumor-associated antigens have been reported that are elevated in the serum of patients with breast cancer. These include a series of antigens related to the human milkfat "globule" membrane family (Burchell *et al.*, *Int. J. Cancer 34:*763-768 (1984); Papsidero *et al.*, *Cancer Res. 44:*4653-4657 (1984); Linsley *et al.*, *Cancer Res. 46*:5444-5450 (1986); Kufe *et al.*, *Hybridoma 3:*223-232 (1984); Hilkens *et al.*, In *Protides of the Biological Fluids*, (Peeters, H., (ed.)), pp. 651-653, Pergamon Press, Oxford, U.K. (1984); Bray *et al.*, *Cancer Res. 47:*5853-5860 (1987); Hilkens *et al.*, In: *Monoclonal Antibodies and Breast Cancer*, (Ceriani, R.L.(ed.)), pp. 28-42, Martinus Nijhoff, Boston, MA, U.S.A. (1985); Linsley *et al.*, *Cancer Res. 48:*2138-2148 (1988)), TAG 72 which is recognized by MAb B72.3 (Gero *et al.*, *J. Clin. Lab. Anal. 3:*360-369 (1989)), and MCA which is recognized by MAb b 12 (Bombardieri *et al.*, *Cancer 63:*490-495 (1989)). The biochemical characterization of these antigens has shown that all of them are heavily glycosylated, high molecular weight glycoproteins with mucin-like properties that are expressed on the surface of, and are shed or secreted by tumor cells. Comparison of these antigens with 90K indicates that the latter is distinct from the previously described antigens. This conclusion is supported by the fact that its electrophoretic migration is unaffected by neuraminidase digestion, suggesting that it is an unsialilated molecule which lacks 0-glycosidically linked oligosaccharides which are typical of mucins (data not shown) (Gahmberg *et al.*, *Eur. J. Biochem. 122:*581-586 (1982)).

Other tumor associated antigens have been described that migrate in SDS:PAGE as molecules of Mr 90,000 daltons. We have distinguished these antigens and the 90K antigen. The antigen recognized by MAb B6.2 (Kufe *et al.*, *Cancer Res.* *43*:851-857 (1983); Schlom *et al.*, *Cancer 54*:2777-2794 (1984)) is a cell surface glycoprotein and, unlike 90K, is highly restricted to breast cancer cells. The melanoma-associated antigen termed p97, gp87, or gp95 (Brown *et al.*, *J. Immunol. 127*:539-546 (1981); Dippold *et al.*, *Proc. Natl. Acad. Sci. USA 77*:6114-6118 (1980); Liao *et al.*, *J. Cell. Biochem. 27*:303-316 (1985)) is a membrane protein which is structurally related to transferrin (Brown *et al.*, *Nature 296*:171-173 (1982)). Another melanoma antigen, FD, is also a surface glycoprotein the expression of which is restricted to a very limited number of cells (Mattes *et al.*, *Cancer Res. 47*:6614-6619 (1987)). Finally, the antigen defined by MAb 3G2-C6 (Zhang *et al.*, *Cancer Res. 49*:6621-6628 (1989)) is a surface component which is expressed in a significant number of bladder cancers but only marginally in breast cancer (Young *et al.*, *Cancer Res. 45*:4439-4446 (1985)).

Example 2

Cloning Of The 90K Gene

End terminal sequencing of the 90K antigen revealed the following amino acid sequence (SEQ ID NO:3): Val Asn Asp Gly Asp Met Arg Leu Ala Asp Gly Gly Ala Thr Asn Gln Gly Arg Val Glu Ile Phe. Based on this amino acid sequence, a "guessmer" of 66 nucleotides was designed on the basis of codon usage frequencies (Lathe, J., *Mol. Biol. 183*:1-12 (1985)) using the amino-terminal sequence: VNDGDM(S)LADGGATNQGRVEIF (SEQ ID NO:4). The nucleotide sequence (SEQ ID NO:5) utilized was as follows:

5′ GTG AAT GAT GGC GAC ATG TCC CTG
GCT GAT GGC GGC GCC ACC AAC CAG
GGC CGG GTG GAG ATC TTC 3′.

The guessmer or nucleic acid probe was ^{32}P end-labeled and was used to screen a λgt10 library prepared from MCF7 polyA^{+} RNA (complexity: $5x10^{5}$). Techniques of nucleic acid hybridization in clone identification are disclosed by Maniatis *et al.* and Sambrook *et al.* (both entitled: *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1982 and 1989, respectively)) and by Hames *et al.*, in *Nucleic Acid Hybridization, A Practical Approach,* IRL Press, Washington, D.C. (1985), which references are herein incorporated by reference.

Positive phages were isolated including two *Eco*RI inserts of ~1,200 bp and ~900 bp. The complete insert was then cloned utilizing the *Eco*RI partial inserts. The DNA fragments were cloned into the Bluescript® plasmid (Stratagene, La Jolla, CA). The insert size was approximately 2,206 nucleotides.

Sequence analyses of the original clones and subclones were performed according to the methods of Sanger *et al.* *(Proc. Natl. Acad. Sci. USA 74*:5463 (1977)) and Maxam *et al.* *(Proc. Natl. Acad. Sci. USA 74*:560 (1977)).

The protein sequence was revealed to be 585 amino acids, 1,755 nucleotides. A 5′ leader of 131 nucleotides and a 3′ trailer of 320 nucleotides was found. The complete nucleotide and projected amino acid sequence is given in Figure 1 (SEQ ID NO:1 AND SEQ ID NO:2, respectively). Included in Table 3 are Northern blot analyses of RNAs from tumors and normal tissues.

Example 3

Cell Culture and Stable Expression of the 90K Antigen

Materials and Methods

Construction of an IR-95 Expression Plasmid. Using standard protocols, a 2147 bp *ClaI/XhoI* cDNA-fragment was subcloned into the eukaryotic, cytomegalovirus promoter-based expression vector (pCMVNEO-IR95) (Figure 8) containing expression units for mouse dihydrofolate reductase (DHFR) cDNA and the bacterial neomycin phosphotransferase (*neo*) gene for amplification and selection, respectively.

Cell Culture. Human BT-20 breast tumor cells (American Type Culture Collection, Rockville, MD, USA, Deposit Number HTB 19) were grown in RPMI 1640 (GIBCO, Gaithersburg, MD) supplemented with 3% FCS, 2 mM L-glutamine and antibiotics in a humidified CO_2 incubator. Selection for neomycin resistance after electroporation of the pCMVNEO-IR95 plasmid was performed in the same medium.

Electroporation. Exponentially growing BT 20 cells were washed twice with PBS, were harvested by trypsinization and were pelleted. The pellet was washed three times with PBS. The cells were resuspended in PBS at a concentration of approximately 5 x 10^6 cells/ml. Electroporation was performed with the Gene Pulser Transfection apparatus from Bio-Rad Laboratories, Segrate, Italy. For stabie expression, 0.8 ml of cell suspension was mixed with 20 μg of linearized plasmid DNA and 50 μg of sheared Salmon sperm DNA in an electroporation cuvette. A single pulse of increasing field strength (240-270 V) was delivered from a 500 μF capacitor at room temperature. After the pulse and a 10 minute incubation on ice, the cells were transferred to the non-selective media as above. The Trypan blue exclusion test was used for determining the viability of the cells at 10 minutes after electroporation during the mock electroporations.

Selection and Amplification. Two days after electroporation, the cells were passaged into selective medium containing Geneticin (G418, GIBCO, Gaithersburg, MD) at 400 μg/ml. Clones were picked using metal cloning cylinders with petroleum jelly for the bottom seal. The clones were expanded and cultured in 12 well clusters (Costar, Cambridge, MA) in Alpha-MEM (GIBCO, Cat. #072-01900A) containing 3% FCS, glutamine (2 mM) and antibiotics plus methotrexate (Sigma Chemical Co., St. Louis, MO, U.S.A.) at concentrations of 10 and 50 μM. After methotrexate selection, the cells were cultured in DMEM high glucose (GIBCO, Gaithersburg, MD) supplemented with 3% FCS, 2 mM glutamine, 50 μg/ml Gentamicin and 1 μM Methotrexate.

(^{35}S)Methionine Labeling and Immunoprecipitation. Subconfluent cells in 6 well clusters (Nunc) were washed with 1 ml of PBS twice and were grown overnight in 1 ml of methionine free DMEM/0.5% ULTROSOR-G containing 50 μCi (1 Ci = 37 GBq) of (^{35}S)methionine. For immunoprecipitation, conditioned media was briefly spun and was mixed with 1 μg/ml aprotinin and 1 μg/ml leupeptin. Protein A-Sepharose (Pharmacia, Uppsala, Sweden) was washed thrice with PBS and 30 μl (1:1) suspension mixed with 2 μg of MAb SP-2 and was incubated for 30 minutes at room temperature. The protein A-Sepharose-SP-2 complex was washed three times with HNTG buffer (20 mM HEPES, pH 7.5/150 mM NaCl/10% glycerol/0.1% Triton X-100) and was incubated with conditioned media for 2 hours at 4°C. Protein A-Sepharose beads were washed three times with HNTG buffer. Moist beads were suspended in 30 μl of 1 x SDS gel-loading buffer, were boiled for 3 minutes at 100°C and were immediately chilled on ice. The proteins were separated on 10% SDS-polyacrylamide gel and were analyzed by autoradiography.

Results

For expression of this protein, a cDNA coding for the entire 585-amino acid polypeptide was placed under the transcriptional control of the cytomegalovirus early promoter. In addition, the expression vector contained the *neo* resistance gene, which conferred cellular resistance to the aminoglycoside antibiotic G418 and therefore allowed selection of primary transfectants, as well as the DHFR gene for methotrexate resistance, which was used to select for cells containing amplified transfected DNA sequences. Bacterial plasmid sequences, including an origin of replication and the gene for ampicillin resistance, allowed replication of the entire expression plasmid in *E. coli*. Figure 9 shows the autoradiogram of immunoprecipitates of the first three stable clones. The intensities of the bands are reflective of the relative amounts of protein secreted by each clone.

Example 4

Transient Expression of the 90K Antigen

Materials and Methods

Construction of Expression Plasmid. Using standard protocols (Sambrook *et al., Molecular Cloning, A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA (1989) Vols. 1-3) the expression plasmid was constructed by introducing a 2147 bp Cla (position 726 in Bluescript II KS - Xho (position 2118 in Figure 1) restriction fragment into the eukaryotic, cytomegalovirus promoter-based expression vector pCMV (Figure 7).

Transient Expression. Human embryonic kidney 293 fibroblasts (American Type Culture Collection, Rockville, MD, USA, Deposit Number CRL 1573) were grown in DMEM containing 10% FCS and antibiotics.

One day prior to transfection, 2×10^5 cells were seeded into each well of a six-well dish. Transfections were carried out according to the protocol of Chen and Okayama *Mol. Cell. Biol.* 7:2745-2752 (1987) with a total of 4 μg of CsCl gradient-purified plasmid-DNA/well. Sixteen hours after the addition of precipitates, the cells were washed once with DMEM, and fresh growth medium was added.

Metabolic Labeling. For metabolic labeling, the cells were grown overnight with (^{35}S)methionine (50 μCi/ml) in methionine-free DMEM (0.5 ml/well) containing 1% dialyzed FCS.

Tunicamycin Treatment. For blocking the formation of protein N-glycosidic linkages, tunicamycin was added to the medium at a final concentration of 0.1 to 1.0 μg/ml for 16 hours.

Cell Lysis and Immunoprecipitation. The cells were lysed on ice with 0.3 ml of lysis buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride (PMSF), 200 units/ml aprotinin, 10 mM sodium pyrophosphate, and 10 μg/ml leupeptin. The lysates were transferred to microfuge tubes, were vortexed for 10 seconds, and were precleared by centrifugation at 12,500 rpm for 15 minutes at 4°C.

For immunoprecipitation, 10 μl of protein A-sepharose (swollen and prewashed in 20 mM HEPES, pH 7.5) and 1 μg MAb SP-2 was added to the cleared lysate and incubated at 4°C for 3 hours. The conditioned medium was used for immunoprecipitation after adding aprotinin (200 units/ml) and PMSF (2 mM final) and preclearing by centrifugation. Precipitates were washed three times with 1 ml of washing buffer (lysis buffer with 0.1% Triton X-100). SDS-sample buffer was added, the samples were boiled and were loaded on SDS-PAGE for the separation of precipitated proteins.

Results

Cells of the transformed 293 cell line were placed into six-well dishes and were transfected with the CMV-expression construct as described above (Figure 10: lanes 1-8). Control cells were transfected with the insertless plasmid pCMV (Figure 10: lanes 7 and 8).

Sixteen hours prior to cell lysis the growth medium was exchanged for labeling medium which contained 50 μCi/ml (^{35}S)methionine. For the same incubation period tunicamycin was added at a final concentration of 0.1 μg/ml (Figure 10: lanes 3 and 4) or 1.0 μg/ml (Figure 10: lanes 5 and 6).

Both the cell lysate (L) and the conditioned medium (M) were used for immunoprecipitations with MAb SP-2. Precipitated proteins were separated on a 8.5% SDS-PAGE. Figure 10 shows the autoradiograph of a 20 hour exposure of the dried gel.

Immunoprecipitation with MAb SP-2 from the conditioned media of the adenovirus type 5-(Ad 5)-transformed cell line 293 resulted in the appearance of a single band at 95 Kd (lane 8). A corresponding signal was not detectable (lane 9) in immunoprecipitates of the cell lysate.

Using the conditioned media, transiently expressing cells (cells transfected with the CMV-expression plasmid carrying the cDNA-insert) resulted in a several fold increase in signal intensity of the 95 kd band (Figure 10: lane 2). At the same time, a protein of approximately 77 kd was detectable in immunoprecipitates of the corresponding cell lysates (Figure 10: lane 1). Tunicamycin treatment of transiently expressing cells reduced the signal intensity for both the 95 kd protein (lanes 4 and 6) and the 77 kd protein (lanes 3 and 5). The tunicamycin effect was dose dependent.

Example 5

Purification of IR-95

IR-95 was also purified using the thiophilic sepharose chromatography method described below.

Materials

Thiophilic Sepharose (AFFI-T)
Metal Chelate Sepharose
Protein A- Sepharose
Amm. Sulphate
Sod. Sulphate
Copper Sulphate
Glycine
Sod. Phosphate, Dibasic Anhydrous
Potassium Chloride
Sod. Chloride
Hank's balanced salt solution (GIBCO)

Buffers

1. Buffer A: For 1 litre; Sod. Chloride 13 gm, Pot. Chloride 0.2 gm, Sod. Phosphate Dibasic, Anhydrous 1.6 gm, Sod. Sulphate 0.5 M and EDTA, 1 mM pH of the buffer titrated to 8.2.
2. Buffer B: For 1 litre; Sod. Chloride 13 gm, Pot. Chloride 0.2 gm, Sod. Phosphate Dibasic, Anhydrous 1.6 gm, Sod. Sulphate 0.3 M and EDTA, 1 mM pH of the buffer titrated to 8.2.
3. Buffer C: For 1 litre; Sod. Chloride 13 gm, Pot. Chloride 0.2 gm, Sod. Phosphate Dibasic, Anhydrous 1.6 gm, and EDTA, 1 mM pH of the solution titrated to 8.2.

4. Buffer D: For 1 litre; Sod. Phosphate Dibasic, Anhydrous 7.098 gm and Sod. Chloride 5.8 gm pH of the solution titrated to 8.

5. Buffer E: For 1 litre; Sod. Phosphate Dibasic, Anhydrous 7.098 gm, Glycine 100 mM and Sod. Chloride 5.8 gm pH of the solution titrated to 8.

Step 1: Thiophilic Sepharose Chromatography

Thiophilic Sepharose chromatography consisted of the following steps:

A- Ammonium Sulphate Precipitation. Preclarified conditioned medium was concentrated ten fold on a hollow fibre ultrafilteration cartridge (40 KD, Nunc). Concentrated medium was precipitated, with solid ammonium sulphate to 42% saturation (assuming the maximum saturation at 533 gm/litre). Ammonium sulphate was added slowly and pH was titrated back to approximately 8.0 by using dilute ammonium hydroxide. Let the solution stir overnight.

In case the conditioned media is not concentrated, the precipitation should be done with solid Amm. sulphate to 42% saturation.

B- Centrifugation. Ammonium sulphate precipitate was centrifuged at 8000 rpm in a GS3 rotor (Sorvall). The supernatant was discarded and the pellet was dissolved using a 10X volume in buffer A.

C- Thiophilic Sepharose Batch Elution. The required volume of the thiophilic sepharose (Kem-En-Tec, Copenhagen, Denmark) was extensively washed with water on a sintered glass funnel using mild suction (removes the sodium azide). The matrix was aspirated until the cracks appeared in the bed. Five bed volumes of buffer A was then passed through it while stirring lightly with a glass rod to get ride of the trapped air in the matrix. The protein solution from the previous step was passed through the matrix under mild suction without letting it dry. The protein solution was recycled three times. The matrix was washed with 50 to 100 bed volumes with buffer A with

occasional stirring. The matrix was then washed with 50 to 100 volumes of buffer B with occasional stirring without letting it dry. The thiophilic sepharose was eluted with 10 bed volumes of buffer C adding one bed volume at a time and lastly with sterile water. After the last bed volume was added, the matrix was aspirated to dryness.

The eluates were pooled and precipitated with 70% ammonium sulphate and stirred for at least four hours in the cold room. The precipitate was collected by centrifugation at 10000 rpm and dissolved in buffer D.

D. Dialysis. The protein solution was dialysed against buffer D for at least four hours in the cold room with two changes of buffer.

Step 2: Metal Chelate Chromatography

The metal chelate chromatography was carried out as described below:

Equiliberation and Column Elution. Metal chelate sepharose (Pharmacia) was packed in a glass column under gravity to a packed volume of 4 ml. Matrix was washed extensively with water to remove ethanol. A copper sulphate solution (10 mg per ml) was passed over the matrix. Normally 10 ml of the copper sulphate solution is enough for lading of the matrix. The matrix was again washed with 10 to 20 column volumes of water to remove the excess copper sulphate. Then the matrix was washed with 10 column volumes of buffer E and equiliberated with 20 column volumes of buffer D.

The dialysed protein solution was centrifuged at 10000 rpm to get rid of the coagulated protein. The protein solution was diluted five fold in the equiliberation buffer and passed over the matrix twice. The matrix washed with 50 column volumes of the equiliberation buffer and protein was eluted using a linear gradient of 20 column volumes each of buffer D and buffer E at a flow rate of 1 ml per minute. Normally, the protein elutes from the column in the second peak. Active fractions were pooled and concentrated on

Centricon-30. The activity of purified protein was checked by immunoprecipitation.

Step 3: Immunoprecipitation

Purified protein was checked for its ability to be immunoprecipitated with SP-2 monoclonal antibody. 50 μl of 1:1 suspension of Protein A-Sepharose was washed three times with one ml of buffer C by brief spinning and aspirations. Two μg of SP-2 MAb plus protein sample were rotated for two hours in the cold room. The beads were washed three times with one ml of buffer C by repeated centrifugation and aspirations. In the end, the beads were aspirated and moist beads lysed in 1X Laemeli buffer and electrophoresed.

Step 4: Storage

The purified protein was buffer exchanged and concentrated with Hank's balanced salt solution using Centricon-30 to 2-3 mg/ml and mixed with one volume of 2 M glucose before freezing at -20 degrees.

Example 6

Enhancement of Natural Killer (NK)
and Lymphokine Activated Killer (LAK) Cell Activity

Peripheral blood mononuclear cells (PBL) were isolated from fresh heparinized blood by Ficoll-Hypaque gradient centrifugation after partial depletion of monocytes by adherence to plastic surfaces (45 min, 37°C). PBL at the concentration of $2x10^6$ cells/ml were cultured in RMPI-1640 medium supplemented with 10% heat-inactivated fetal calf serum and antibiotics. Purified IR-95 was added in various concentrations (50 ng/ml to 2000 ng/ml) for 16 h. As a control, PBL were incubated in the same culture conditions for

the same period of time without IR-95. At the end of the incubation period, cells were washed and tested as effector cells in the short term (4 h) ^{41}Cr-release cytotoxicity assay (Coligan, J.E. *et al.*, *Current Protocols in Immunology*, Green Publishing Associates and Wiley Interscience, New York (1992)) against target cells, i.e. K562 cells for NK activity and Daudi cells for LAK activity at an effector:target ratio of 1:40. Data points are averages of five different experiments performed in quadruplicate. Spontaneous ^{51}Cr-release was 15% of the total in all cases. IR-95 at concentrations in the range of 500-2000 ng/ml for 16 hours markedly increases both NK and LAK cytotoxic activity (Figure 11).

All publications and patent applications mentioned in this specification are indicative of the level of skill of one in the art to which this invention pertains. All publications and patent applications are hereby incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims. Modifications of the above-described modes for carrying out the invention that are obvious to persons of skill in the art, such as those in the fields of medicine, immunology, hybridoma technology, pharmacology, and/or related fields, are intended to be within the scope of the following claims.

The hybridoma cell line which produces MAb SP-2 referred to on page 6 at lines 5 to 10 has also been deposited (under the Budapest Treaty) on 5 February 1993 at DeutscheSammlung von Mikrooganismen and Zellkulturen GmbH (DSM) in Braunschweig, Germany, under accession number DSM ACC2116.

Table 1. Purification of the 90K Antigen from:

Source: Treatment thereof	Protein (mg)	Activity (Units) x 10^{-6})	Yield (%)	Purification (fold)	Specific Activity (Units/mg)
CG-5 tissue culture fluid (10 liters)	14,100	2.51	100	1	0.18
:$(NH_4)_2$ SO_4 precipitate	3,700	2.61	104	4	0.7
:Sepharose CL-6B eluate	230	1.92	76.4	46.8	8.3
:DEAE-cellulose eluate	61	1.71	68.1	157	28
:Immunoaffinity eluate	0.029	0.44	17.5	84,300	15,174
Breast Cancer Serum (50 ml)	3,100	1.28	100	1	0.41
:$(NH_4)_2$ SO_4 precipitate	950	1.38	106	3.5	1.4
:Sepharose CL-6B eluate	58	0.91	71	38	15.6
:DEAE-cellulose eluate	15	0.71	55.4	115	47.3
:Immunoaffinity eluate	0.013	0.28	21.8	52,277	15,538
Ovarian Cancer Ascitic Fluid: (1 liter)	13,500	0.62	100	1	45.9
:$(NH_4)_2$ SO_4 precipitate	4,250	0.71	114	3.6	167
:Sepharose CL-6B eluate	282	0.58	93.5	44.7	2,056
:DEAE-cellulose eluate	64	0.53	85.4	180	8,281
:Immunoaffinity eluate	0.11	0.21	34	83,380	19,090

Table 2. Effects of Chemical and Physical Treatments on 90K Activity.

Treatment	Relative Binding Activity
Control	1
Methanol	0.04
Guanidine-HC1, 6M	0.18
Urea, 6M	0.19
SDS	0.14
Dithiothreitol	0.89
2-mercaptoethanol	1.1
Iodacetic acid	0.93
NP-40	1.11
Tween-20	1.05
Triton X-100	0.88
Lyophilization	0
Heat (100°C, 5 min)	0
Periodate, 0 mM	1
Periodate, 10 mM	1.05
Periodate, 20 mM	0.91
Periodate, 30 mM	0.95
Periodate, 40 mM	0.90
Periodate, 50 mM	0.71

Table 3:
Northern Blot Analyses of RNAs from Tumors and Normal Tissues

Mammary Carcinomas	
Total tested:	70
90K positive:	50 (71%)
Leukemias	
Total tested:	8
90K positive:	8 (100%)
Melanoma cell lines	
Total tested:	9
90K positive:	9 (100%)
Normal Tissues	
placenta	+
brain	+
muscle	+
spleen	+
kidney	+
liver	+
fetal liver	+
breast	+/-
thyroid	+/-
bladder	+
skeletal muscle	+/-
skin	+/-
ovary	+
duodenum	+
colon	+
small intestine	+
myometrium	+
stomach	+
pancreas	+
adrenals	+
lung	+

Table 4. Amino Acid Composition of the 90K Antigen

	Molar Percentage		
Amino Acid	CG-5 Cells	Breast Cancer Serum	Ovarian Cancer Ascitic Fluid
Glu/Gln	11.8	10.7	11.1
Asp/Asn	7.6	6.9	8.3
Ser	12.4	11.9	11.9
Thr	4.3	4.8	4.3
Gly	8.8	9.1	8.9
Pro	5.1	4.9	4.7
Val	4.9	4.2	5.1
Leu	12.1	13.3	13.2
Ile	1.1	0.9	1.3
Ala	8.1	7.9	6.9
Phe	2.8	2.4	2.5
Met	1.1	1.3	0.9
His	3.1	3.3	2.9
Lys	2.5	2.7	2.8
Arg	4.1	3.9	3.2
Tyr	3.5	3.7	3.7
Trp	N.D.	N.D.	N.D.
Cys	N.D.	N.D.	N.D.

N.D. = Not determined

Table 5. Distribution of Serium IR-95 Levels in Different Pathophysiological Conditions				
Group	No. of Subjects	Mean +/- SD (units/ml)	No. of Cases With Increased 90K Levels vs. Normal (%)	
Healthy controls	165	1.1 +/- 0.3	10	(6)
Cancer	297	1.9 +/- 1.7	77	(26)
HIV infection	63	2.7 +/- 1.2	43	(69)
Hepatitis B virus infection	87	2.2 +/- 1.7	35	(40)
Epstein Barr virus infection	21	2.7 +/- 2.1	7	(33)
Autoimmune disease	28	1.8 +/- 0.9	10	(36)
Hemodialysis	19	1.6 +/- 0.8	5	(26)
Down syndrome	12	2.2 +/- 1.6	4	(33)
Pregnancy	18	1.8 +/- 0.7	18	(100)
Aging (>85 years)	29	1.5 +/- 0.4	8	(27)

Circulating serum IR-95 concentrations (unit/ml) were determined by a solid-phase, enzyme-linked, immunoabsorbent procedure that uses mAb SP-2 as the coating antibody. Levels of more than 1.75 units/ml (normal mean +/- 2SD) were considered positive determinations. The serum level of IR-95 was not affected by sex and blood group.

A total of 214 serum samples were obtained from the following categories of patients attending the Chieti Uniiversity Hospital: Hepatitis B virus infection (69 cases), Epstein Barr virus infection (21 cases), autoimmune disease (15 rheumatoid arthritis, 7 systemic lupus erythematosus, 6 autoimmune uveitis), hemodialysis (19 cases), Down syndrome (12 cases). In addition, serum samples were obtained from 18 women at different periods of gestation and 29 apparently healthy subjects of more than 85 years of age.

Cut off value of serum IR-95 is 1.7 units/ml (mean +/- 2 SD).

All means for different groups of subjects were significantly greater than those for healthy controls (p = 0.0001, analysis of variance).

SEQUENCE LISTING

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(ii) TITLE OF INVENTION: Genetic Sequences For A 90K Tumor-Associated Antigen, IR-95

(iii) NUMBER OF SEQUENCES: 5

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(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US (to be assigned)
(B) FILING DATE: Herewith
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: IT RM 92A000100
(B) FILING DATE: 17-FEB-1992

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2206 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 132..1886

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CACGCTCCAT ACTGGGAGAG GCTTCTGGGT CAAAGGACCA GTCTGCAGAG GGATCCTGTG      60

GCTGGAAGCG AGGAGGCTCC ACACGGCCGT TGCAGCTACC GCAGCCAGGA TCTGGGCATC     120

CAGGCACGGC C ATG ACC CCT CCG AGG CTC TTC TGG GTG TGG CTG CTG GTT      170
             Met Thr Pro Pro Arg Leu Phe Trp Val Trp Leu Leu Val
               1               5                  10

GCA GGA ACC CAA GGC GTG AAC GAT GGT GAC ATG CGG CTG GCC GAT GGG       218
Ala Gly Thr Gln Gly Val Asn Asp Gly Asp Met Arg Leu Ala Asp Gly
     15                  20                  25

GGC GCC ACC AAC CAG GGC CGC GTG GAG ATC TTC TAC AGA GGC CAG TGG       266
Gly Ala Thr Asn Gln Gly Arg Val Glu Ile Phe Tyr Arg Gly Gln Trp
 30                  35                  40                  45

GGC ACT GTG TGT GAC AAC CTG TGG GAC CTG ACT GAT GCC AGC GTC GTC       314
Gly Thr Val Cys Asp Asn Leu Trp Asp Leu Thr Asp Ala Ser Val Val
                 50                  55                  60

TGC CGG GCC CTG GGC TTC GAG AAC GCC ACC CAG GCT CTG GGC AGA GCT       362
Cys Arg Ala Leu Gly Phe Glu Asn Ala Thr Gln Ala Leu Gly Arg Ala
             65                  70                  75

GCC TTC GGG CAA GGA TCA GGC CCC ATC ATG CTG GAC GAG GTC CAG TGC       410
Ala Phe Gly Gln Gly Ser Gly Pro Ile Met Leu Asp Glu Val Gln Cys
         80                  85                  90

ACG GGA ACC GAG GCC TCA CTG GCC GAC TGC AAG TCC CTG GGC TGG CTG       458
Thr Gly Thr Glu Ala Ser Leu Ala Asp Cys Lys Ser Leu Gly Trp Leu
     95                 100                 105

AAG AGC AAC TGC AGG CAC GAG AGA GAC GCT GGT GTG GTC TGC ACC AAT       506
Lys Ser Asn Cys Arg His Glu Arg Asp Ala Gly Val Val Cys Thr Asn
110                 115                 120                 125

GAA ACC AGG AGG CAC CCA CAC CCT GGA CCT CTC CAG GGA GCT CTC GGA       554
Glu Thr Arg Arg His Pro His Pro Gly Pro Leu Gln Gly Ala Leu Gly
                130                 135                 140

GCC CTT GGC CAG ATC TTT GAC AGC CAG CGG GGC TGC GAC CTG TCC ATC       602
Ala Leu Gly Gln Ile Phe Asp Ser Gln Arg Gly Cys Asp Leu Ser Ile
            145                 150                 155

AGC GTG AAT GTG CAG GGC GAG GAC GCC CTG GGC TTC TGT GGC CAC ACG       650
Ser Val Asn Val Gln Gly Glu Asp Ala Leu Gly Phe Cys Gly His Thr
        160                 165                 170

GTC ATC CTG ACT GCC AAC CTG GAG GCC CAG GCC CTG TGG AAG GAG CCG       698
Val Ile Leu Thr Ala Asn Leu Glu Ala Gln Ala Leu Trp Lys Glu Pro
    175                 180                 185

GGC AGC AAT GTC ACC ATG AGT GTG GAT GCT GAG TGT GTG CCC ATG GTC       746
Gly Ser Asn Val Thr Met Ser Val Asp Ala Glu Cys Val Pro Met Val
190                 195                 200                 205

AGG GAC CTT CTC AGG TAC TTC TAC TCC CGA AGG ATT GAC ATC ACC CTG       794
Arg Asp Leu Leu Arg Tyr Phe Tyr Ser Arg Arg Ile Asp Ile Thr Leu
                210                 215                 220

TCG TCA GTC AAG TGC TTC CAC AAG CTG GCC TCT GCC TAT GGG GCC AGG       842
Ser Ser Val Lys Cys Phe His Lys Leu Ala Ser Ala Tyr Gly Ala Arg
            225                 230                 235

CAG CTG CAG GGC TAC TGC GCA AGC CTC TTT GCC ATC CTC CTC CCC CAG       890
Gln Leu Gln Gly Tyr Cys Ala Ser Leu Phe Ala Ile Leu Leu Pro Gln
        240                 245                 250

GAC CCC TCG TTC CAG ATG CCC CTG GAC CTG TAT GCC TAT GCA GTG GCC       938
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Asp Pro Ser Phe Gln Met Pro Leu Asp Leu Tyr Ala Tyr Ala Val Ala
    255                 260                 265

ACA GGG GAC GCC CTG CTG GAG AAG CTC TGC CTA CAG TTC CTG GCC TGG      986
Thr Gly Asp Ala Leu Leu Glu Lys Leu Cys Leu Gln Phe Leu Ala Trp
270                 275                 280                 285

AAC TTC GAG GCC TTG ACG CAG GCC GAG GCC TGG CCC AGT GTC CCC ACA     1034
Asn Phe Glu Ala Leu Thr Gln Ala Glu Ala Trp Pro Ser Val Pro Thr
                290                 295                 300

GAC CTG CTC CAA CTG CTG CTG CCC AGG AGC GAC CTG GCG GTG CCC AGC     1082
Asp Leu Leu Gln Leu Leu Leu Pro Arg Ser Asp Leu Ala Val Pro Ser
            305                 310                 315

GAG CTG GCC CTA CTG AAG GCC GTG GAC ACC TGG AGC TGG GGG GAG CGT     1130
Glu Leu Ala Leu Leu Lys Ala Val Asp Thr Trp Ser Trp Gly Glu Arg
        320                 325                 330

GCC TCC CAT GAG GAG GTG GAG GGC TTG GTG GAG AAG ATC CGC TTC CCC     1178
Ala Ser His Glu Glu Val Glu Gly Leu Val Glu Lys Ile Arg Phe Pro
    335                 340                 345

ATG ATG CTC CCT GAG GAG CTC TTT GAG CTG CAG TTC AAC CTG TCC CTG     1226
Met Met Leu Pro Glu Glu Leu Phe Glu Leu Gln Phe Asn Leu Ser Leu
350                 355                 360                 365

TAC TGG AGC CAC GAG GCC CTG TTC CAG AAG AAG ACT CTG CAG GCC CTG     1274
Tyr Trp Ser His Glu Ala Leu Phe Gln Lys Lys Thr Leu Gln Ala Leu
                370                 375                 380

GAA TTC CAC ACT GTG CCC TTC CAG TTG CTG GCC CGG TAC AAA GGC CTG     1322
Glu Phe His Thr Val Pro Phe Gln Leu Leu Ala Arg Tyr Lys Gly Leu
            385                 390                 395

AAC CTC ACC GAG GAT ACC TAC AAG CCC CGG ATT TAC ACC TCG CCC ACC     1370
Asn Leu Thr Glu Asp Thr Tyr Lys Pro Arg Ile Tyr Thr Ser Pro Thr
        400                 405                 410

TGG AGT GCC TTT GTG ACA GAC AGT TCC TGG AGT GCA CGG AAG TCA CAA     1418
Trp Ser Ala Phe Val Thr Asp Ser Ser Trp Ser Ala Arg Lys Ser Gln
    415                 420                 425

CTG GTC TAT CAG TCC AGA CGG GGG CCT TTG GTC AAA TAT TCT TCT GAT     1466
Leu Val Tyr Gln Ser Arg Arg Gly Pro Leu Val Lys Tyr Ser Ser Asp
430                 435                 440                 445

TAC TTC CAA GCC CCC TCT GAC TAC AGA TAC TAC CCC TAC CAG TCC TTC     1514
Tyr Phe Gln Ala Pro Ser Asp Tyr Arg Tyr Tyr Pro Tyr Gln Ser Phe
                450                 455                 460

CAG ACT CCA CAA CAC CCC AGC TTC CTC TTC CAG GAC AAG AGG GTG TCC     1562
Gln Thr Pro Gln His Pro Ser Phe Leu Phe Gln Asp Lys Arg Val Ser
            465                 470                 475

TGG TCC CTG GTC TAC CTC CCC ACC ATC CAG AGC TGC TGG AAC TAC GGC     1610
Trp Ser Leu Val Tyr Leu Pro Thr Ile Gln Ser Cys Trp Asn Tyr Gly
        480                 485                 490

TTC TCC TGC TCC TCG GAC GAG CTC CCT GTC CTG GGC CTC ACC AAG TCT     1658
Phe Ser Cys Ser Ser Asp Glu Leu Pro Val Leu Gly Leu Thr Lys Ser
    495                 500                 505

GGC GGC TCA GAT CGC ACC ATT GCC TAC GAA AAC AAA GCC CTG ATG CTC     1706
Gly Gly Ser Asp Arg Thr Ile Ala Tyr Glu Asn Lys Ala Leu Met Leu
510                 515                 520                 525

TGC GAA GGG CTC TTC GTG GCA GAC GTC ACC GAT TTC GAG GGC TGG AAG     1754
Cys Glu Gly Leu Phe Val Ala Asp Val Thr Asp Phe Glu Gly Trp Lys
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                 530                 535                 540

GCT GCG ATT CCC AGT GCC CTG GAC ACC AAC AGC TCG AAG AGC ACC TCC     1802
Ala Ala Ile Pro Ser Ala Leu Asp Thr Asn Ser Ser Lys Ser Thr Ser
            545                 550                 555

TCC TTC CCC TGC CCG GCA GGG CAC TTC AAC GGC TTC CGC ACG GTC ATC     1850
Ser Phe Pro Cys Pro Ala Gly His Phe Asn Gly Phe Arg Thr Val Ile
        560                 565                 570

CGC CCC TTC TAC CTG ACC AAC TCC TCA GGT GTG GAC TAGACGCGTG          1896
Arg Pro Phe Tyr Leu Thr Asn Ser Ser Gly Val Asp
    575                 580                 585

GCCAAGGGTG GTGAGAACCG GAGAACCCCA GGACGCCCTC ACTGCAGGCT CCCCTCCTCG   1956

GCTTCCTTCC TCTCTGCAAT GACCTTCAAC AACCGGCCAC CAGATGTCGC CCTACTCACC   2016

TGAGGCTCAG CTTCAAGAAA TTACTGGAAG GCTTCCACTA GGGTCCACCA GGAGTTCTCC   2076

CACCACCTCA CCAGTTTCCA GGTGGTAAGC ACCAGGAGGC CCTCGAGGTT GCTCTGGATC   2136

CCCCCACAGC CCCTGGTCAG TCTGCCCTTG TCACTGGTCT GAGGTCATTA AAATTACATT   2196

GAGGTTCCTA                                                          2206
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(2) INFORMATION FOR SEQ ID NO:2:

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     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 585 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Thr Pro Pro Arg Leu Phe Trp Val Trp Leu Leu Val Ala Gly Thr
  1               5                  10                  15

Gln Gly Val Asn Asp Gly Asp Met Arg Leu Ala Asp Gly Gly Ala Thr
             20                  25                  30

Asn Gln Gly Arg Val Glu Ile Phe Tyr Arg Gly Gln Trp Gly Thr Val
         35                  40                  45

Cys Asp Asn Leu Trp Asp Leu Thr Asp Ala Ser Val Val Cys Arg Ala
     50                  55                  60

Leu Gly Phe Glu Asn Ala Thr Gln Ala Leu Gly Arg Ala Ala Phe Gly
 65                  70                  75                  80

Gln Gly Ser Gly Pro Ile Met Leu Asp Glu Val Gln Cys Thr Gly Thr
                 85                  90                  95

Glu Ala Ser Leu Ala Asp Cys Lys Ser Leu Gly Trp Leu Lys Ser Asn
            100                 105                 110

Cys Arg His Glu Arg Asp Ala Gly Val Val Cys Thr Asn Glu Thr Arg
        115                 120                 125

Arg His Pro His Pro Gly Pro Leu Gln Gly Ala Leu Gly Ala Leu Gly
    130                 135                 140

Gln Ile Phe Asp Ser Gln Arg Gly Cys Asp Leu Ser Ile Ser Val Asn
145                 150                 155                 160

Val Gln Gly Glu Asp Ala Leu Gly Phe Cys Gly His Thr Val Ile Leu
```

165 170 175

Thr Ala Asn Leu Glu Ala Gln Ala Leu Trp Lys Glu Pro Gly Ser Asn
180 185 190

Val Thr Met Ser Val Asp Ala Glu Cys Val Pro Met Val Arg Asp Leu
195 200 205

Leu Arg Tyr Phe Tyr Ser Arg Arg Ile Asp Ile Thr Leu Ser Ser Val
210 215 220

Lys Cys Phe His Lys Leu Ala Ser Ala Tyr Gly Ala Arg Gln Leu Gln
225 230 235 240

Gly Tyr Cys Ala Ser Leu Phe Ala Ile Leu Leu Pro Gln Asp Pro Ser
245 250 255

Phe Gln Met Pro Leu Asp Leu Tyr Ala Tyr Ala Val Ala Thr Gly Asp
260 265 270

Ala Leu Leu Glu Lys Leu Cys Leu Gln Phe Leu Ala Trp Asn Phe Glu
275 280 285

Ala Leu Thr Gln Ala Glu Ala Trp Pro Ser Val Pro Thr Asp Leu Leu
290 295 300

Gln Leu Leu Leu Pro Arg Ser Asp Leu Ala Val Pro Ser Glu Leu Ala
305 310 315 320

Leu Leu Lys Ala Val Asp Thr Trp Ser Trp Gly Glu Arg Ala Ser His
325 330 335

Glu Glu Val Glu Gly Leu Val Glu Lys Ile Arg Phe Pro Met Met Leu
340 345 350

Pro Glu Glu Leu Phe Glu Leu Gln Phe Asn Leu Ser Leu Tyr Trp Ser
355 360 365

His Glu Ala Leu Phe Gln Lys Lys Thr Leu Gln Ala Leu Glu Phe His
370 375 380

Thr Val Pro Phe Gln Leu Leu Ala Arg Tyr Lys Gly Leu Asn Leu Thr
385 390 395 400

Glu Asp Thr Tyr Lys Pro Arg Ile Tyr Thr Ser Pro Thr Trp Ser Ala
405 410 415

Phe Val Thr Asp Ser Ser Trp Ser Ala Arg Lys Ser Gln Leu Val Tyr
420 425 430

Gln Ser Arg Arg Gly Pro Leu Val Lys Tyr Ser Ser Asp Tyr Phe Gln
435 440 445

Ala Pro Ser Asp Tyr Arg Tyr Tyr Pro Tyr Gln Ser Phe Gln Thr Pro
450 455 460

Gln His Pro Ser Phe Leu Phe Gln Asp Lys Arg Val Ser Trp Ser Leu
465 470 475 480

Val Tyr Leu Pro Thr Ile Gln Ser Cys Trp Asn Tyr Gly Phe Ser Cys
485 490 495

Ser Ser Asp Glu Leu Pro Val Leu Gly Leu Thr Lys Ser Gly Gly Ser
500 505 510

Asp Arg Thr Ile Ala Tyr Glu Asn Lys Ala Leu Met Leu Cys Glu Gly
515 520 525

Leu Phe Val Ala Asp Val Thr Asp Phe Glu Gly Trp Lys Ala Ala Ile

```
            530                 535                 540

Pro Ser Ala Leu Asp Thr Asn Ser Ser Lys Ser Thr Ser Ser Phe Pro
545                 550                 555                 560

Cys Pro Ala Gly His Phe Asn Gly Phe Arg Thr Val Ile Arg Pro Phe
                565                 570                 575

Tyr Leu Thr Asn Ser Ser Gly Val Asp
            580                 585
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Val Asn Asp Gly Asp Met Arg Leu Ala Asp Gly Gly Ala Thr Asn Gln
1               5                   10                  15

Gly Arg Val Glu Ile Phe
            20
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Val Asn Asp Gly Asp Met Ser Leu Ala Asp Gly Gly Ala Thr Asn Gln
1               5                   10                  15

Gly Arg Val Glu Ile Phe
            20
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
GTGAATGATG GCGACATGTC CCTGGCTGAT GGCGGCGCCA CCAACCAGGG CCGGGTGGAG    60

ATCTTC                                                               66
```

CLAIMS

1. A DNA segment coding for an IR-95 polypeptide.

2. The DNA segment according to claim 1, wherein the DNA segment has the sequence set forth in SEQ ID NO:1 which encodes the amino acid sequence set forth in SEQ ID NO:2.

3. The DNA segment according to claim 1, wherein the DNA segment has the sequence set forth in SEQ ID NO:1.

4. The DNA segment according to claim 1, wherein the DNA segment encodes the amino acid sequence set forth in SEQ ID NO:2.

5. The DNA segment according to claim 1, wherein said IR-95 has the terminal amino acid sequence set forth in SEQ ID NO:3.

6. A recombinant DNA molecule comprising, 5′ to 3′, a promoter effective to initiate transcription in a host cell and the DNA segment according to claim 1.

7. A cell that contains the DNA molecule according to claim 6.

8. A recombinant DNA molecule comprising a vector and the DNA segment according to claim 1.

9. The recombinant DNA molecule according to claim 8, wherein said vector is an expression vector.

10. A cell that contains the DNA molecule according to claim 9.

11. A cell that contains the DNA molecule according to claim 10.

12. A method of producing IR-95 or fragment thereof, comprising:
    (a) providing a DNA molecule comprising expressible sequences encoding said IR-95 or fragment thereof;
    (b) transforming a host with said DNA molecule;
    (c) expressing said IR-95 or fragment sequences of said DNA molecule in said host; and
    (d) isolating said IR-95, or fragment thereof, which is produced by said expression.

13. The method according to claim 12, wherein said DNA molecule has the nucleotide sequence as shown in SEQ ID NO:1 which encodes the amino acid sequence set forth in SEQ ID NO:2.

14. The method according to claim 12, wherein said DNA molecule has the nucleotide sequence as shown in SEQ ID NO:1.

15. The method according to claim 12, wherein said DNA molecule encodes the amino acid sequence set forth in SEQ ID NO:2.

16. The method according to claim 12, wherein said DNA molecule codes for IR-95 which has the terminal amino acid sequence set forth in SEQ ID NO:3.

CACGCTCCATACTGGGAGAGGCTTCTGGGTCA 32
AAGGACCAGTCTGCAGAGGGATCCTGTGGCTGGAAGCGAGGAGGCTCCACACGGCCGTTGCAGCTACCGCAGCCAGGATCTGGGCATCCAGGCACGGCC 131

```
M   T   P   P   R   L   F   W   V   W   L   L   V   A   G   T   Q   G   V   N   D   G   D   M   R      25
ATG ACC CCT CCG AGG CTC TTC TGG GTG TGG CTG CTG GTT GCA GGA ACC CAA GGC GTG AAC GAT GGT GAC ATG CGG   206

L   A   D   G   G   A   T   N   Q   G   R   V   E   I   F   Y   R   G   Q   W   G   T   V   C   D      50
CTG GCC GAT GGG GGC GCC ACC AAC CAG GGC CGC GTG GAG ATC TTC TAC AGA GGC CAG TGG GGC ACT GTG TGT GAC   281

N   L   W   D   L   T   D   A   S   V   V   C   R   A   L   G   F   E   N   A   T   Q   A   L   G      75
AAC CTG TGG GAC CTG ACT GAT GCC AGC GTC GTC TGC CGG GCC CTG GGC TTC GAG AAC GCC ACC CAG GCT CTG GGC   356

R   A   A   F   G   Q   G   S   G   P   I   M   L   D   E   V   Q   C   T   G   T   E   A   S   L     100
AGA GCT GCC TTC GGG CAA GGA TCA GGC CCC ATC ATG CTG GAC GAG GTC CAG TGC ACG GGA ACC GAG GCC TCA CTG   431

A   D   C   K   S   L   G   W   L   K   S   N   C   R   H   E   R   D   A   G   V   V   C   T   N     125
GCC GAC TGC AAG TCC CTG GGC TGG CTG AAG AGC AAC TGC AGG CAC GAG AGA GAC GCT GGT GTG GTC TGC ACC AAT   506

E   T   R   R   H   P   H   P   G   P   L   Q   G   A   L   G   A   L   G   Q   I   F   D   S   Q     150
GAA ACC AGG AGG CAC CCA CAC CCT GGA CCT CTC CAG GGA GCT CTC GGA GCC CTT GGC CAG ATC TTT GAC AGC CAG   581

R   G   C   D   L   S   I   S   V   N   V   Q   G   E   D   A   L   G   F   C   G   H   T   V   I     175
CGG GGC TGC GAC CTG TCC ATC AGC GTG AAT GTG CAG GGC GAG GAC GCC CTG GGC TTC TGT GGC CAC ACG GTC ATC   656

L   T   A   N   L   E   A   Q   A   L   W   K   E   P   G   S   N   V   T   M   S   V   D   A   E     200
CTG ACT GCC AAC CTG GAG GCC CAG GCC CTG TGG AAG GAG CCG GGC AGC AAT GTC ACC ATG AGT GTG GAT GCT GAG   680

C   V   P   M   V   R   D   L   L   R   Y   F   Y   S   R   R   I   D   I   T   L   S   S   V   K     225
TGT GTG CCC ATG GTC AGG GAC CTT CTC AGG TAC TTC TAC TCC CGA AGG ATT GAC ATC ACC CTG TCG TCA GTC AAG   806

C   F   H   K   L   A   S   A   Y   G   A   R   Q   L   Q   G   Y   C   A   S   L   F   A   I   L     250
TGC TTC CAC AAG CTG GCC TCT GCC TAT GGG GCC AGG CAG CTG CAG GGC TAC TGC GCA AGC CTC TTT GCC ATC CTC   881

L   P   Q   D   P   S   F   Q   M   P   L   D   L   Y   A   Y   A   V   A   T   G   D   A   L   L     275
CTC CCC CAG GAC CCC TCG TTC CAG ATG CCC CTG GAC CTG TAT GCC TAT GCA GTG GCC ACA GGG GAC GCC CTG CTG   956

E   K   L   C   L   Q   F   L   A   W   N   F   E   A   L   T   Q   A   E   A   W   P   S   V   P     300
GAG AAG CTC TGC CTA CAG TTC CTG GCC TGG AAC TTC GAG GCC TTG ACG CAG GCC GAG GCC TGG CCC AGT GTC CCC  1031

T   D   L   L   Q   L   L   L   P   R   S   D   L   A   V   P   S   E   L   A   L   L   K   A   V     325
ACA GAC CTG CTC CAA CTG CTG CTG CCC AGG AGC GAC CTG GCG GTG CCC AGC GAG CTG GCC CTA CTG AAG GCC GTG  1106
```

FIG.1A

D T W S W G E R A S H E E V E G L V E K I R F P M 350

GAC ACC TGG AGC TGG GGG GAG CGT GCC TCC CAT GAG GAG GTG GAG GGC TTG GTG GAG AAG ATC CGC TTC CCC ATG 1181

M L P E E L F E L Q F N L S L Y W S H E A L F Q K 375

ATG CTC CCT GAG GAG CTC TTT GAG CTG CAG TTC AAC CTG TCC CTG TAC TGG AGC CAC GAG GCC CTG TTC CAG AAG 1256

K T L Q A L E F H T V P F Q L L A R Y K G L N L T 400

AAG ACT CTG CAG GCC CTG GAA TTC CAC ACT GTG CCC TTC CAG TTG CTG GCC CGG TAC AAA GGC CTG AAC CTC ACC 1331

E D T Y K P R I Y T S P T W S A F V T D S S W S A 425

GAG GAT ACC TAC AAG CCC CGG ATT TAC ACC TCG CCC ACC TGG AGT GCC TTT GTG ACA GAC AGT TCC TGG AGT GCA 1406

R K S Q L V Y Q S R R G P L V K Y S S D Y F Q A P 450

CGG AAG TCA CAA CTG GTC TAT CAG TCC AGA CGG GGG CCT TTG GTC AAA TAT TCT TCT GAT TAC TTC CAA GCC CCC 1481

S D Y R Y Y P Y Q S F Q T P Q H P S F L F Q D K R 475

TCT GAC TAC AGA TAC TAC CCC TAC CAG TCC TTC CAG ACT CCA CAA CAC CCC AGC TTC CTC TTC CAG GAC AAG AGG 1556

V S W S L V Y L P T I Q S C W N Y G F S C S S D E 500

GTG TCC TGG TCC CTG GTC TAC CTC CCC ACC ATC CAG AGC TGC TGG AAC TAC GGC TTC TCC TGC TCC TCG GAC GAG 1631

L P V L G L T K S G G S D R T I A Y E N K A L M L 525

CTC CCT GTC CTG GGC CTC ACC AAG TCT GGC GGC TCA GAT CGC ACC ATT GCC TAC GAA AAC AAA GCC CTG ATG CTC 1706

C E G L F V A D V T D F E G W K A A I P S A L D T 550

TGC GAA GGG CTC TTC GTG GCA GAC GTC ACC GAT TTC GAG GGC TGG AAG GCT GCG ATT CCC AGT GCC CTG GAC ACC 1781

N S S K S T S S F P C P A G H F N G F R T V I R P 575

AAC AGC TCG AAG AGC ACC TCC TCC TTC CCC TGC CCG GCA GGG CAC TTC AAC GGC TTC CGC ACG GTC ATC CGC CCC 1856

F Y L T N S S G V D 585

TTC TAC CTG ACC AAC TCC TCA GGT GTG GAC TAGACGCGTGGCCAAGGGTGGTGAGAACCGGAGAACCCCAGGACGCCCTCACTGCAGGC 1945

TCCCCTCCTCGGCTTCCTTCCTCTCTGCAATGACCTTCAACAACCGGCCACCAGATGTCGCCCTACTCACCTGAGGCTCAGCTTCAAGAAATTACTGGA 2044

AGGCTTCCACTAGGGTCCACCAGGAGTTCTCCCACCACCTCACCAGTTTCCAGGTGGTAAGCACCAGGAGGCCCTCGAGGTTGCTCTGGATCCCCCCAC 2143

AGCCCCTGGTCAGTCTGCCCTTGTCACTGGTCTGAGGTCATTAAAATTACATTGAGGTTCCTA 2206

FIG.1B

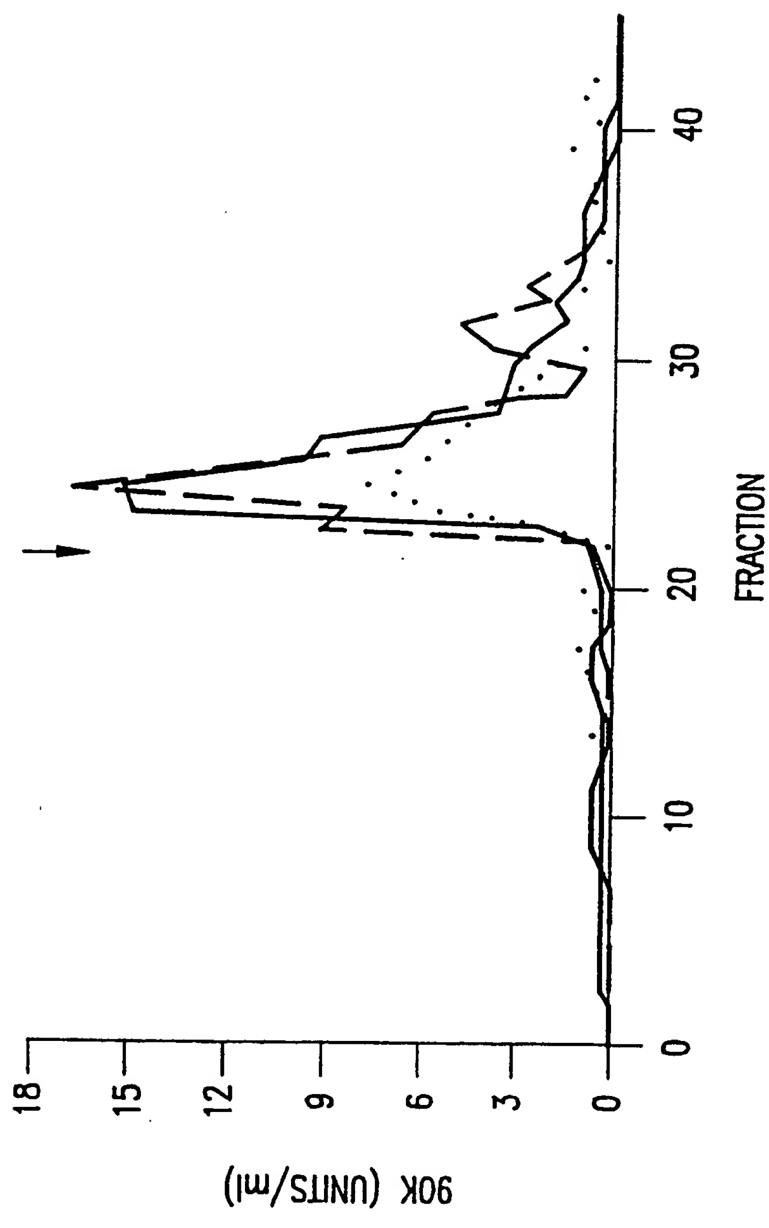
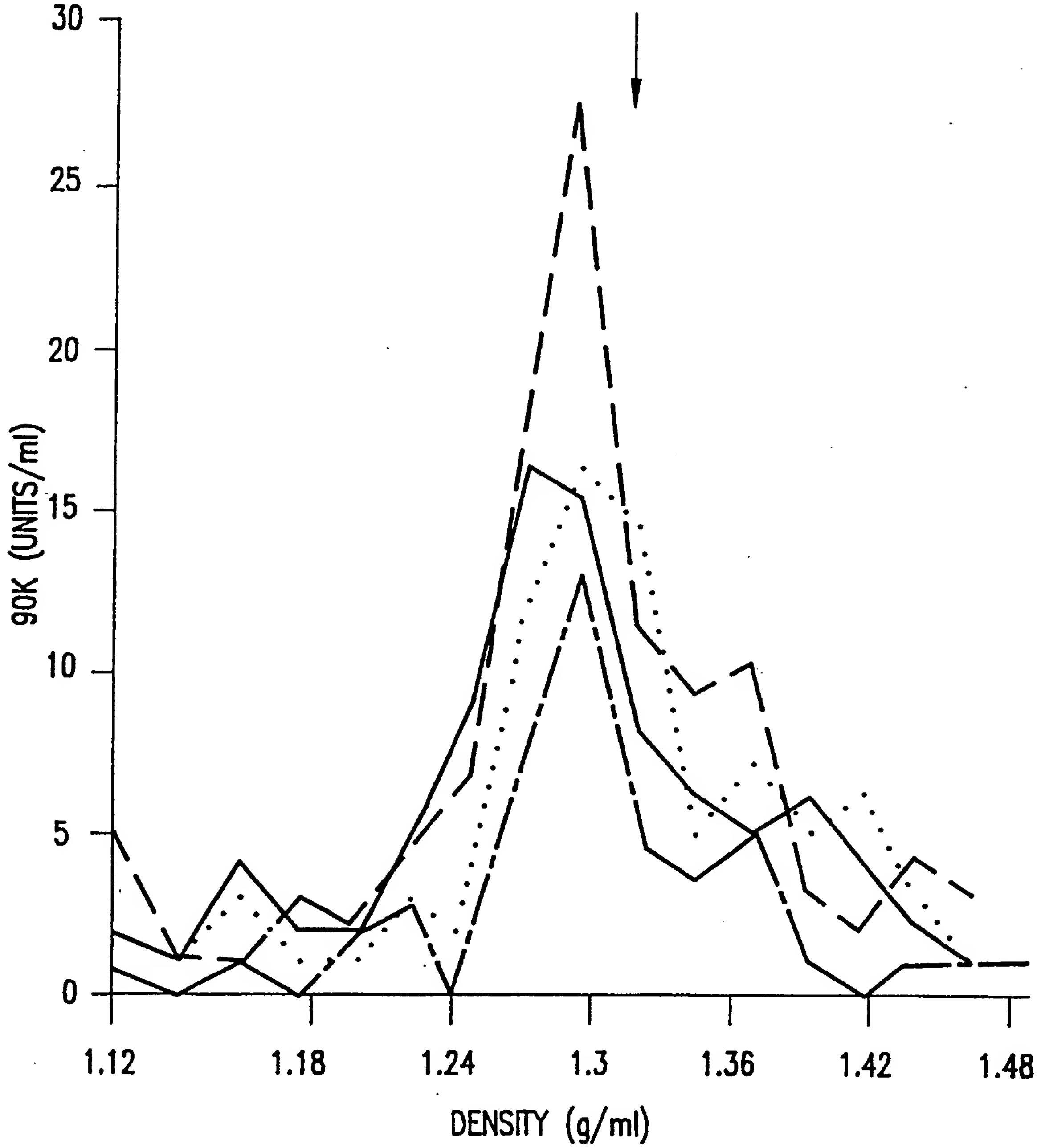


FIG.2



30
25
20
15
10
5
0
90K (UNITS/ml)
1.12
1.18
1.24
1.3
1.36
1.42
1.48
DENSITY (g/ml)

FIG.3

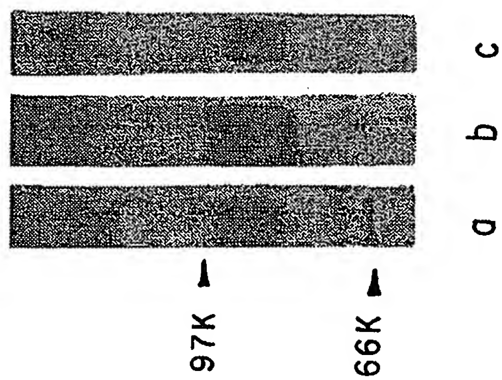


FIG.4B

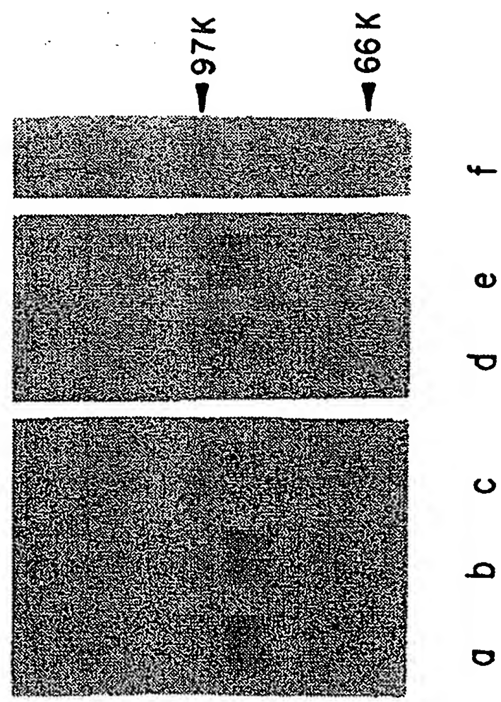


FIG.4A

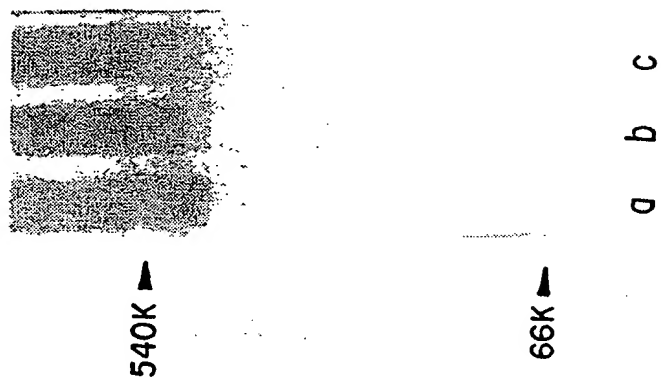


FIG. 5B

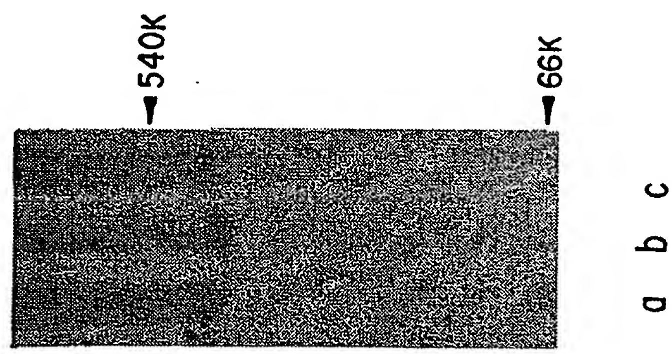


FIG. 5A

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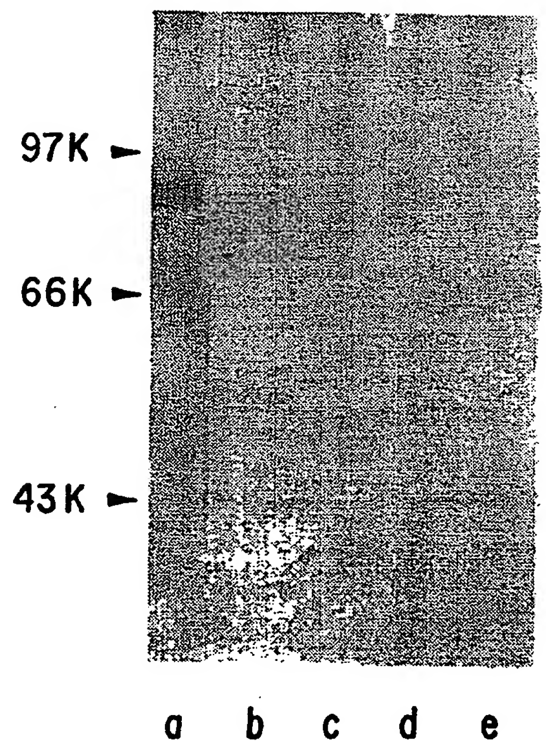


FIG. 6A

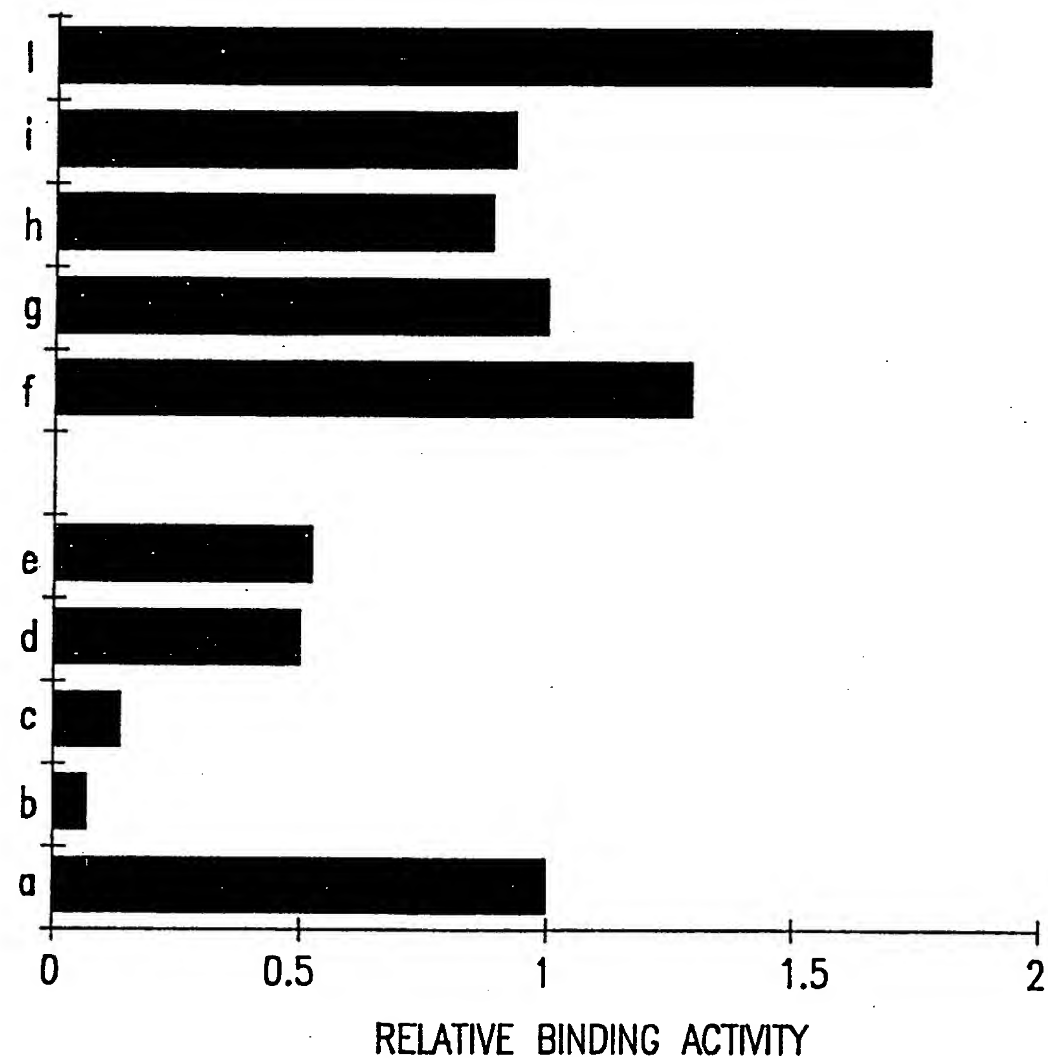
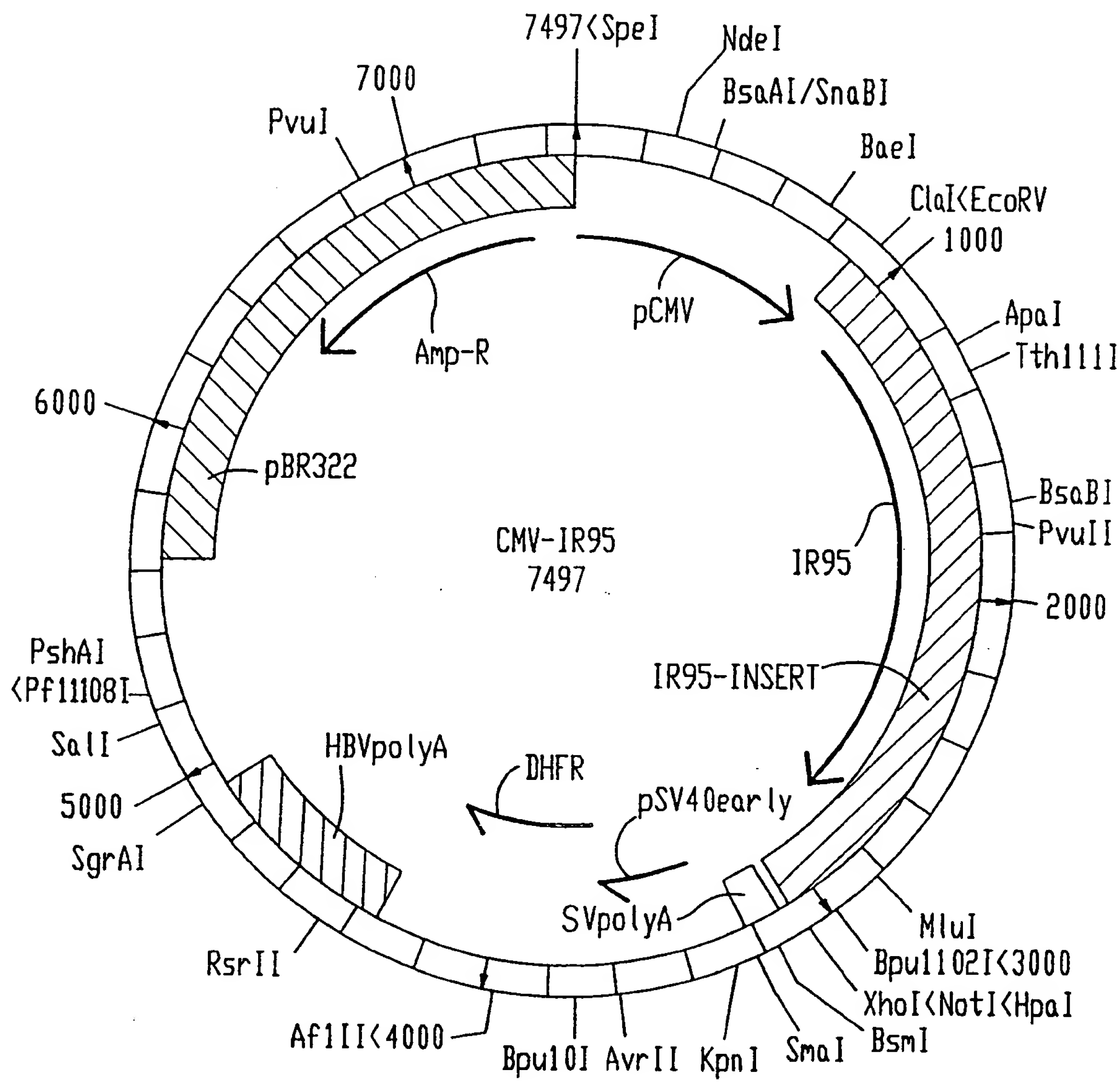


FIG.6B


7497<SpeI
NdeI
BsaAI/SnaBI
BaeI
ClaI<EcoRV
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ApaI
Tth111I
BsaBI
PvuII
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MluI
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XhoI<NotI<HpaI
BsmI
SmaI
KpnI
AvrII
Bpu10I
AflII<4000
RsrII
SgrAI
5000
SalI
PshAI
<Pfl1108I
6000
pBR322
PvuI
7000
Amp-R
pCMV
IR95
IR95-INSERT
pSV40early
SVpolyA
DHFR
HBVpolyA
CMV-IR95
7497

FIG.7

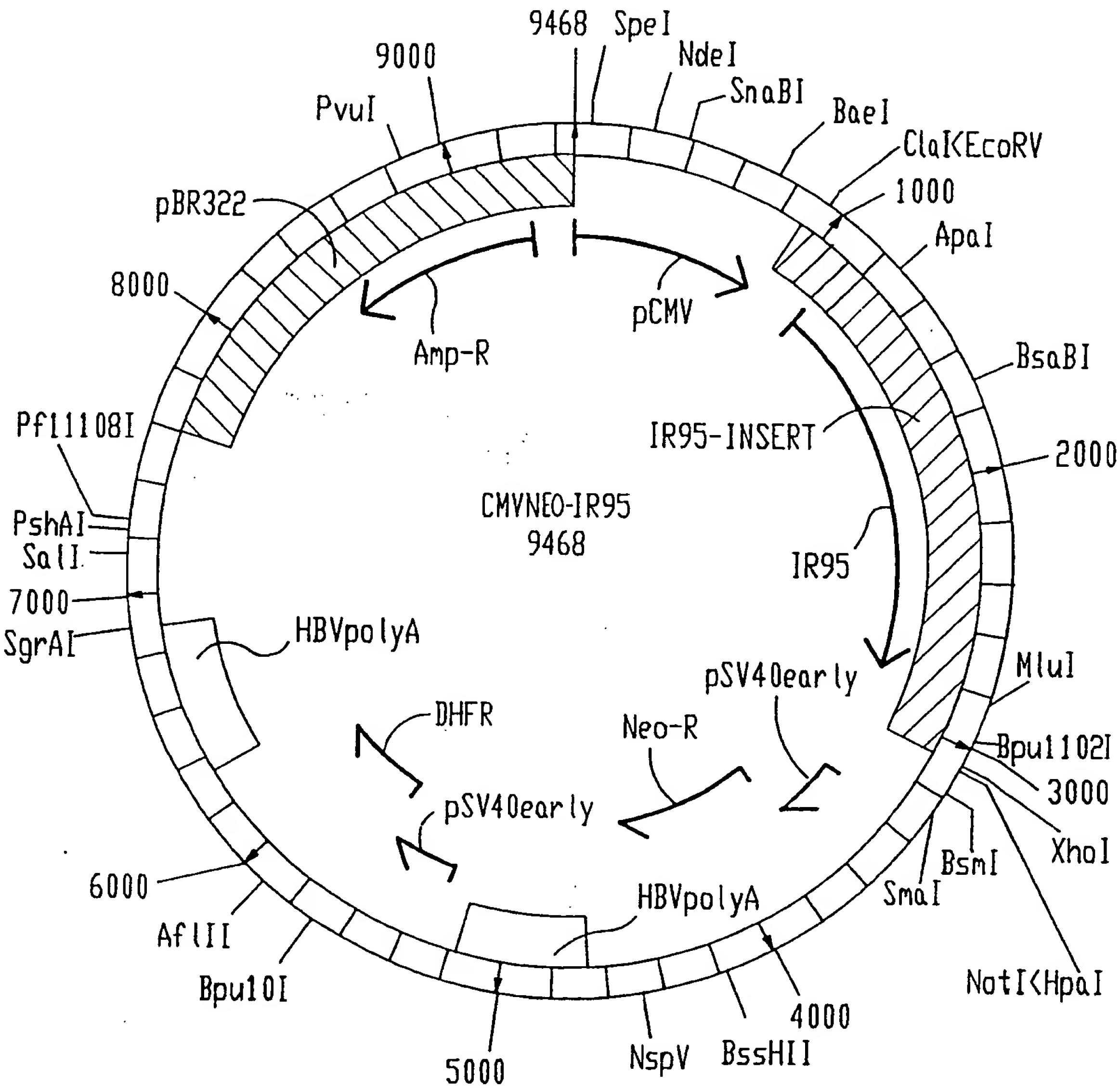
9468
SpeI
NdeI
SnaBI
BaeI
ClaI<EcoRV
1000
ApaI
BsaBI
2000
MluI
Bpu1102I
3000
XhoI
BsmI
SmaI
NotI<HpaI
4000
BssHII
NspV
5000
Bpu10I
AflII
6000
SgrAI
7000
SalI
PshAI
Pfl1108I
8000
pBR322
PvuI
9000
Amp-R
pCMV
IR95-INSERT
IR95
CMVNEO-IR95
9468
HBVpolyA
DHFR
Neo-R
pSV40early
pSV40early
HBVpolyA

FIG.8

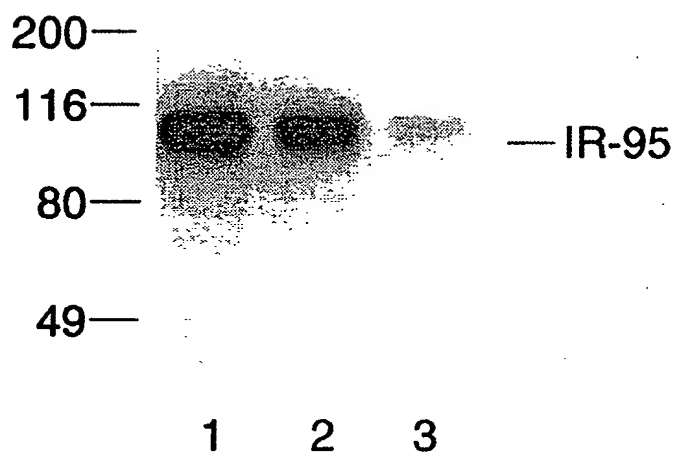

FIG.9

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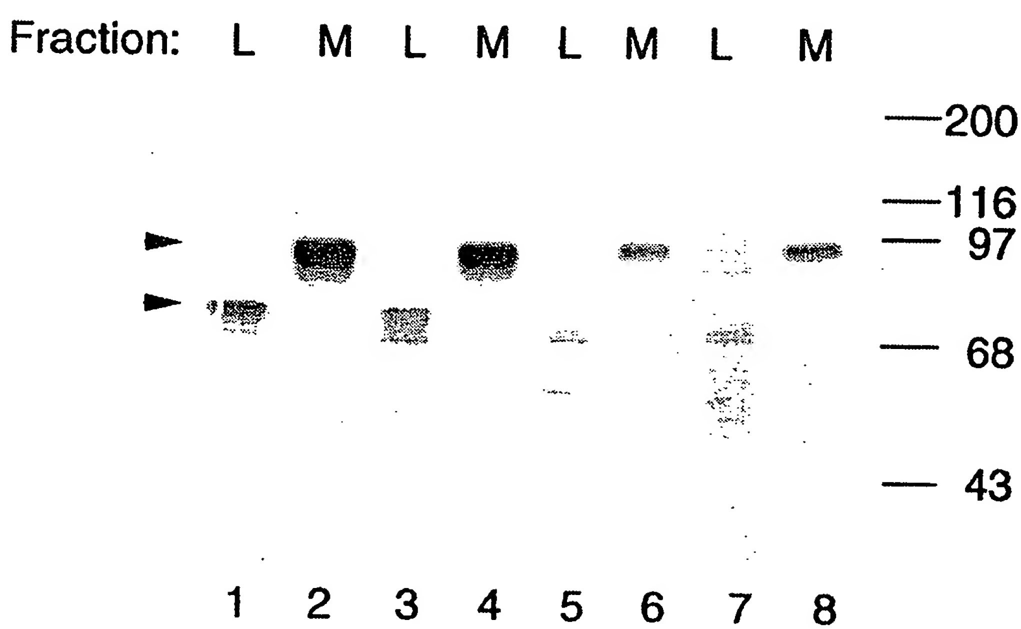

FIG. 10

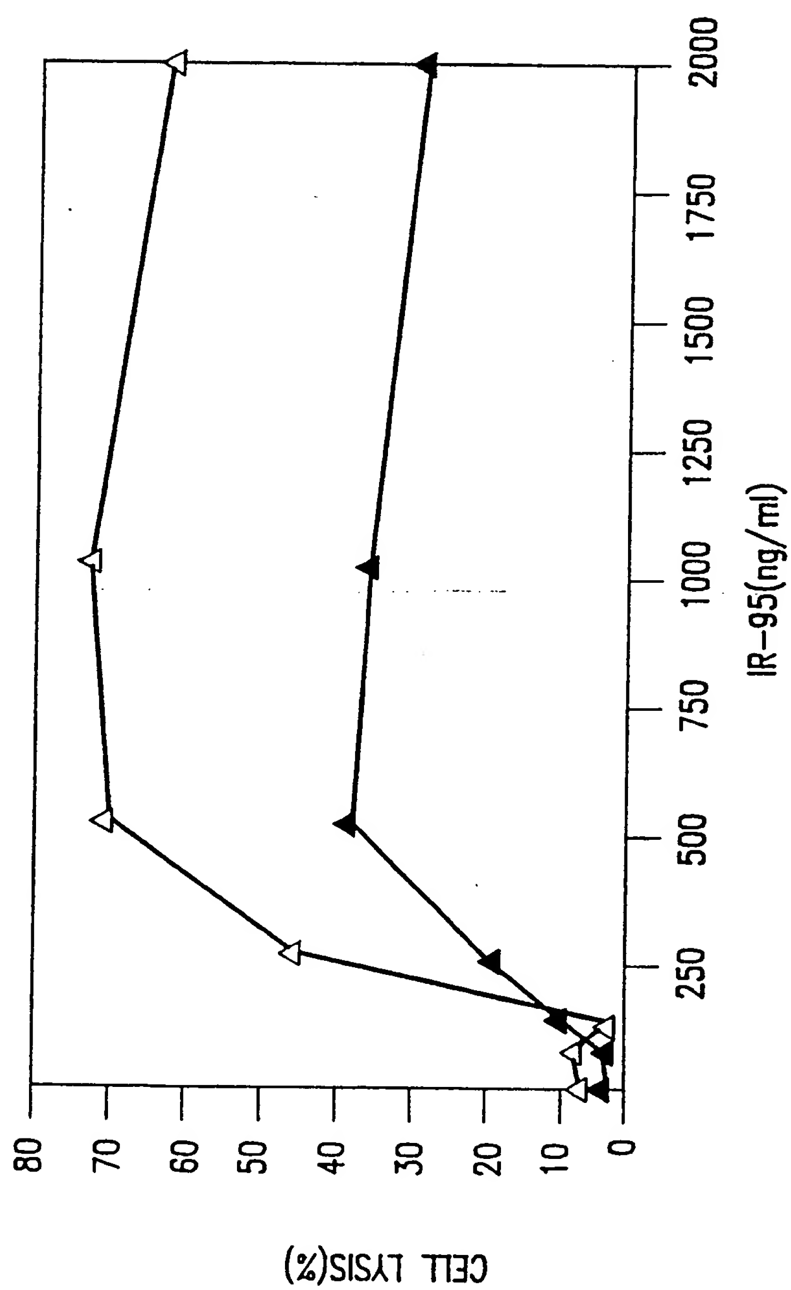
CELL LYSIS(%)
0
10
20
30
40
50
60
70
80
IR-95(ng/ml)
250
500
750
1000
1250
1500
1750
2000

FIG.11

Applicant's or agent's file reference number W/P1708 WO | International appl. PCT/EP 93 / 00382

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 39, line 25-29.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

DeutscheSammlung von Mikroorganismen und Zellkulturen GmbH

Address of depositary institution *(including postal code and country)*

Mascheroder Weg 16, D-3300 Braunschweig, Germany

Date of deposit	Accession Number
5 February 1993	DSM ACC2116

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

In respect of all designated states in which such action is possible and to the extent that it is legally permissible under the law of the designated state, it is requested that a sample of the deposited micro-organism be made available only by the issue thereof to an independent expert in accordance with the relevant patent legislation, e.g. EPC Rule 28(4), U.K. Rule 17(3), Australian Regulation 3.25(3) and generally similar provisions *mutatis mutandis* for any other designated state.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

All designated states

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer C.A.J.A. PASCHE	Authorized officer

Applicant's or agent's file reference number W/P17089WO

International application No. PCT/EP 93/00382

REC'D 12 MAR 1993
WIPO PCT

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 6, line 5-10.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet [X]

Name of depositary institution

Institut Pasteur, Collection Nationale de Cultures de Microorganisms

Address of depositary institution *(including postal code and country)*

28 Rue de Docteur Roux, 75724 Paris Cedex 15, France

Date of deposit	Accession Number
12 April 1991	I-1083

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet [ ]

In respect of all designated states in which such action is possible and to the extent that it is legally permissible under the law of the designated state, it is requested that a sample of the deposited micro-organism be made available only by the issue thereof to an independent expert in accordance with the relevant patent legislation, e.g. EPC Rule 28(4), U.K. Rule 17(3), Australian Regulation 3.25(3) and generally similar provisions *mutatis mutandis* for any other designated state.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

All designated states

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
[X] This sheet was received with the international application	[ ] This sheet was received by the International Bureau on:
Authorized officer C.A.J.A. PASCHE	Authorized officer

Form PCT/RO/134 (July 1992)